Signalling mechanisms involved in TNF-α mediated cytoprotection during ischaemic injury in a C2C12 muscle cell line

by

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Declaration

I, the undersigned, hereby declare that this thesis is my own original work and that all sources have been accurately reported and acknowledged, and that this document has not previously in its entirety nor in part been submitted at any university in order to obtain an academic qualification.

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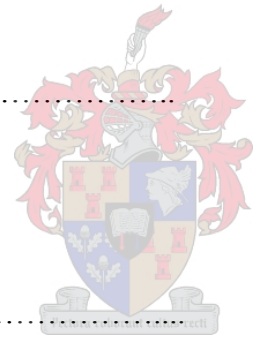

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Abstract

Both, the cytokine Tumor Necrosis Factor-α (TNF-α) and the enzyme cytosolic phospholipase A_2 ($cPLA_2$) are crucial driving forces in mediating the cellular inflammatory response and are involved in ischaemic injury. During an ischaemic insult, TNF-α is endogenously generated. Apart from the recognized effects of TNF-α, such as the induction of apoptosis, proliferation and differentiation, if present in low dosages, it also mediates cytoprotective effects. Upon activation, $cPLA_2$ contributes to the ischaemic challenge with the generation of mediators of cellular injury and apoptosis. Upon stimulation, this calcium dependent enzyme translocates to the phospholipid compartment of the cell membrane and induces the hydrolysis of sn-2 ester bonds in phospholipids. It governs the release of free fatty acids and lysophospholipids and generates role players of inflammation. We suggest a role for $cPLA_2$ in the TNF-α mediated cytoprotection, with a distinct phosphorylation and translocation pattern.

Aims

The involvement of $cPLA_2$ in TNF-α mediated cytoprotection in the C2C12 murine skeletal muscle cell line in tolerance to ischaemia was examined. To investigate the nature of the $cPLA_2$ phosphorylation pattern, the mitogen activated protein kinases (MAPKs) p38 and extracellular regulated kinase (ERK) as contributors to $cPLA_2$ phosphorylation and activation, were examined at appropriate time points. To dissect out the $cPLA_2$ interplay and dependencies with these MAPKs within the pathway context, the selective $cPLA_2$ inhibitor arachidonyl trifluoromethyl ketone ($AACOCF_3$) was employed and its effect on cell viability was examined. Fluorescence microscopy was used to substantiate $cPLA_2$ activation, by assessing its cellular distribution, translocation and cell organelle target preference, using co-localization and z-stack techniques. In addition, the induction of the apoptotic pathway through analysis of caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage was examined. The role of caspase-3 in $cPLA_2$ turnover was addressed employing the caspase inhibitor, Z-DEVD-FMK.

Methods

Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS), and incubated under 5% CO_2 conditions, until 50%-70% confluent. Using DMEM supplemented with 1% horse serum, cell differentiation into myotubes was induced. Differentiated cells were preconditioned for 30 min classically, with 0.5 ng/ml TNF-α or the $cPLA_2$ selective inhibitor $AACOCF_3$ (10 μM) respectively. Followed by a 60 min washout period the cells were subjected to 8 hrs simulated ischaemia. Cellular viability; and $cPLA_2$ phosphorylation- and translocation events were assessed using Western blots and advanced immunocytochemistry and imaging techniques.

Results

Preconditioning with TNF-α, ischaemic preconditioning; and the use of the $cPLA_2$ inhibitor $AACOCF_3$, attenuated the decrease is cell viability brough about by ischaemia. Western blot analysis indicates the induction of the apoptotic pathway with caspase-3 and PARP cleavage. A significantly reduced translocation of $pcPLA_2$ to the nuclear region in the TNF-α preconditioned group compared to the ischaemic group, as reflected by reduced mean nuclear fluorescence intensity, was observed. A z-stack analysis confirmed that the nuclear and endonuclear region was the target organelle for $cPLA_2$. 3-dimensional co-localazation analysis of $pcPLA_2$ with the nuclear marker nucleoporin p62 mirrored these results.

Discussion and conclusion

Our results provide evidence that there is a role for $cPLA_2$ in TNF-α mediated cytoprotection. Although we do not observe a differential activation pattern in terms of $cPLA_2$ phosphorylation at various time points within the ischaemic event, and no differential inactivation of $cPLA_2$ via caspase-mediated $cPLA_2$ cleavage, we describe a differential $cPLA_2$ translocation pattern, similar to that in IPC. Through inhibition of $cPLA_2$ translocation, a functional $cPLA_2$ inhibition might be achieved. This would imply inhibition of the inflammatory pathway and a subsequent reduction in the generation of inflammatory mediators. In addition we describe an effect of TNF-α preconditioning on the efficacy of the caspase inhibitor Z-DEVD-FMK. Our results

shed light on the survival mechanisms employed by the ischaemically challenged cell in a setting of TNF-α mediated cytoprotection. This might lead to novel approaches in the context of inflammation treatment, through agents that control differential $cPLA_2$ trafficking within the cell.

Opsomming

Beide, die sitokien "Tumor Necrosis Factor-α (TNF-α)" en die ensiem, sitosoliese fosfolipase A_2 ($cPLA_2$) is uiters belangrike bemiddelaars van die sellulêre inflammatoriese respons en is verder ook betrokke by isgemiese selskade. TNF-α word endogeen gegenereer tydens 'n isgemiese intervensie. Afgesien van 'n verskeidenheid effekte, soos die inisiëring van apoptose, sel-proliferasie en -differensiasie, bemiddel dit ook selbeskermende meganismes indien dit in lae konsentrasies in die sel teenwoordig is. Na aktivering dra $cPLA_2$ by tot die isgemiese intervensie deur die vorming van bemiddelaars van selskade en apoptose. Hierdie kalsium-afhanklike ensiem translokeer na die fosfolipied membraankomponent na stimulering en induseer die hidrolise van die sn-2 esterbinding in die fosfolipied. Die vrystelling van vry vetsure en lisofosfolipiede word sodoende bewerkstellig wat verder gemetaboliseer kan word tot inflammatoriese bemiddelaars. Ons stel voor dat $cPLA_2$ 'n rol in TNF-α bemiddelde selbeskerming speel en dat dit gepaardgaan met kenmerkende fosforilerings- en translokeringspatrone.

Doelwitte

Die rol van $cPLA_2$ tydens TNF-α bemiddelde selbeskerming is in 'n C2C12 skeletspiersellyn na blootstelling aan isgemie ondersoek. Die rol van die MAPKs, p38 en ERK, is ondersoek om vas te stel of hulle betrokke is by die aktivering van $cPLA_2$. Die selektiewe $cPLA_2$ inhibitor, $AACOCF_3$, is gebruik om te bepaal of die fosforilering van MAPKs ook $cPLA_2$-afhanklik is. Die sellulêre $cPLA_2$ verspreiding, translokering en teiken selorganelle is ook ondersoek met behulp van fluoresensie mikroskopie deur gebruik te maak van ko-lokalisering en z-plaat tegnieke. Verder, is die indusering van die apoptotiese paaie ondersoek deur tegnieke wat kaspase- en PARP kliewing meet. Die kaspase inhibitor, Z-DEVD-FMK, is gebruik om vas te stel of kaspase-3 'n rol speel in $cPLA_2$ kliewing in ons selmodel.

Metodes

Selle is gekweek in Dulbecco's gemodifiseerde Eagles Medium (DMEM) waarby 10% fetale kalf serum (FBS) gevoeg is, en wat geïnkubeer is in 5% CO_2 totdat dit 50%-70% konfluent was. Die selle is verder gedifferensieer in miobuise deur gebruik

te maak van DMEM waarby 1% perdeserum gevoeg is. Gedifferensieerde selle is vir 30 min klassiek geprekondisioneer asook respektiewelik met 0.5 ng/ml TNF-α en die $cPLA_2$ selektiewe inhibitor, $AACOCF_3$ (10 μM). Na ‘n 60 minute uitwas periode is die selle blootgestel aan 8 h gesimuleerde isgemie. Sellulêre lewensvatbaarheid, $cPLA_2$ fosforilering- and translokering is ondersoek deur onderskeidelik gebruik te maak van die “Western” klad metode en gesofistikeerde immunositochemiese beeld tegnieke.

Resultate

Prekondisionering met TNF-α, isgemiese prekondisionering asook inhibisie van as $cPLA_2$ met die inhibitor, $AACOCF_3$, het ‘n beduidende toename in sellewensvatbaarheid tot gevolg gehad. Daar is ook dmv die “Western” klad tegniek bewys dat apoptose geïduseer word deur middel van kaspase-3- en PARP kliewing. Daar is insiggewend minder translokasie van $cPLA_2$ na die nukluêre fraksie in die isgemiese groep in vergelyking met die TNF-α geprekondisioneerde groep waargeneem (die gemiddelde nukluêre fluoreserende intensiteit is bepaal om voorafgaande feit te staaf). Die $cPLA_2$ teiken organel is geverifieer as die nukleus en die endonukluêre gebied deur middel van z-plaat analises. Drie-dimensionele ko-lokaliserings analises van $pcPLA_2$ met die nukluêre merker, nucleoporin p62 het hierdie resultate bevestig.

Bespreking en Gevolgtrekking

Ons resultate verskaf bewyse vir ‘n rol vir $cPLA_2$ in TNF-α bemiddelde selbeskerming. Alhoewel daar nie ‘n differensiële aktiveringspatroon in terme van $cPLA_2$ fosforilering tydens verskeie tydspunte in die isgemiese intervensie waargeneem is nie, en ook geen kaspase-3 bemiddelde kliewing van $cPLA_2$ nie, word ‘n differensiële translokeringspatroon soorgelyk aan die isgemiese prekondisioneringsgroep, waargeneem. Funsksionele $cPLA_2$ inhibisie kan dus moontlik bewerkstellig word deur inhibisie van $cPLA_2$ translokasie. Die inflammatoriese respons kan dus moontlik so inhibieer word en die vorming van minder inflammatoriese bemiddelaars tot gevolg hê. Verder het TNF-α prekondisionering ook ‘n effek op die effektiwiteit van die kaspase-inhibitor, Z-DEVD-FMK. Ons resultate werp ook lig op die meganismes wat deur selle onder

isgemiese toestande uitgeoefen word tydens TNF-α bemiddelde selbeskerming. Hierdie resultate mag lei tot nuwe benaderings in die konteks van behandeling teen inflammasie deur gebruik te maak van middels wat $cPLA_2$ translokering in die sel beheer.

Zusammenfassung

Das Zytokin Tumor Necrosis Factor- α (TNF-α) und das Enzym zytosolische Phospholipase A_2 ($cPLA_2$) sind wichtige Rollenspieler in der zellulären Entzündungsantwort und sind beide involviert in Gwebeischemie. Während einer ischemischen Attacke ist TNF-α in der Zelle generiert. Abgesehen von verschiedenen Effekten, wie die Induction von Apoptosis, Zellteilung und Zelldifferenzierung, wenn anwesend in geringen Konzentrationen, hat TNF-α einen zellschützenden Effekt. $cPLA_2$ nimmt an der ischemischen Herausforderung teil, durch die Herstellung von Mediatoren der Zellverletzung und des apoptotischen Zelltodes. Wenn stimmuliert, translokiert dieses Calcium-abhängige Enzym zu Phospholipidmembrankomponenten und induziert dort die Hydrolisierung von Esterverbindungen im Phospholipid. Es kontrolliert die Freilassung von freien Fettsäuren und Lysophospholipiden und stellt Entzündungsmediatoren durch Arachidonsäure her. Wir stellen eine Rolle für $cPLA_2$, mit einem spezifischen Phosphorilierungs-und Translokierungsmuster im TNFα vermittelten Zellschutz vor.

Ziel

Wir untersuchten die Beteiligung von $cPLA_2$ im TNFα vermittelten Zellschutz in der C2C12 Muskelzelllinie in Toleranz zu Ischemie. Um die Natur des $cPLA_2$ Phosphorilierungsmusters zu beschreiben, wurden die MAPK p38 und ERK als Beisteuerer zur $cPLA_2$ Phosphorilierung und Aktivierung zu bestimmten Zeitpunkten untersucht. Um das Zwischenspiel und die Abhängigkeiten bezüglich der MAPK im Pfadwegkontext zu beurteilen, wurde der selektive $cPLA_2$ Unterdrücker $AACOCF_3$ verwendet, und sein Effekt auf Zellviabilität untersucht. Die Aktivierung von $cPLA_2$ wurde untermalt durch die fluoreszenmikroskopische Beobachtung von zellulärer Verteilung, Translokation und Zielorganelpreffernez, unter Verwendung von Ko-lokalisierungs-und Z-schicht technik. Zusätzlich beurteilten wir die Induktion vom apoptotischen Zelltod, durch die Analysierung von Caspase-3 und PARP Zerteilung. Caspase-3 verwandte Kontrolle über $cPLA_2$ wurde durch die Benutzung vom Caspase Unterdrücker Z-DEVD-FMK addressiert.

Methodik

Zellen wurden in Dulbecos Modified Eagles Medium (DMEM) mit 10% fötalem bovine Serum (FBS) gezüchtet und unter 5 % CO_2 inkubiert, bis eine Konfluenz von 50%-70% erreicht war. Unter Verwendung von DMEM mit 1% Pferdeserum wurde Zelldifferenzierung in Muskeltuben induziert. Differenzierte Zellen wurden für 30 min klassisch, mit 0.5ng/ml TNF-α, oder mit dem selektiven $cPLA_2$ Unterdrücker $AACOCF_3$ (10μM) pre-konditioniert. Gefolgt von einer 60 minütigen Auswaschperiode, wurden die Zellen einer 8 stündigen simulierten Ischemie ausgesetzt. Untersucht wurden Zellviabilität, $cPLA_2$ Phosphorilierungs- und Translokierungsvorgänge unter Verwendung von Western Blot- und vortgeschrittener immunzytochemischen Bild-technik.

Ergebnisse

Pre-Konditionierung mit TNF-α, ischemische Pre-Konditionierung und die Verwendung des $cPLA_2$ Unterdrückers $AACOCF_3$, resultierten in eine signifikante Erhöhung der Zellviabilität. Western blot Analyse erwiesen die Induktion des apoptotischen Pfadweges mit Caspase-3 und PARP Zerteilung. Wir beschreiben eine signifikant reduzierte Translokierung von $pcPLA_2$ zur nukleären Region in der TNF-α-pre-konditionierten Zellgruppe vergichen mit ischemischen Zellen, was in einer reduzierten mitleren nukleären Fluoreszenintensität reflektiert wird. Die Z-schicht Analyse verifiziert eine nukleäre und innernukleäre Region als $cPLA_2$ Zielorganell. Dreidimensionelle Ko-lokalisierungsanalyse von $pcPLA_2$ mit dem nuleären Marker nucleoporin p62 spiegelt diese Ergebnisse wieder.

Diskussion und Schlussfolgerung

Unsere Ergebnisse liefern Beweis für eine Rolle von $cPLA_2$ im TNF-α-vermittelten Zellschutz. Obwohl wir kein differenziertes Aktivierungsmuster beobachten, im Sinne von $cPLA_2$ Phosphorilierung zu verschiedenen Zeitpunkten innerhalb der ischemischen Attacke, und keine differenzielle $cPLA_2$ Inaktivierung durch Caspase vermittelte $cPLA_2$ Zerteilung, so beschreiben wir jedoch ein differenziertes Translokationsmuster, gleichend dem in klassischer Pre-konditionierung. Durch die Unterdrückung der innerzellulären $cPLA_2$ Bewegung könnte eine funktionelle $cPLA_2$ Unterdrückung resultieren. Dies würde eine Unterdrückung des

Enzündungspfadweges implizieren, folgend von einer reduzierten Bildung von Entzündungsmediatoren. Zusätzlich beschreiben wir einen Effekt von TNF-α Pre-konditionierung auf die Effizienz des Caspase Unterdrückers Z-DEVD-FMK. Unsere Ergebnisse bringen Einsichten in den Überlebensmechanismus einer ischemisch herausgeforderten Zelle in der Situation des TNF-α vermittelten Zellschutztes. Dies könnte zu neuen Herangehensweisen im Kontext der Entzündungsbehandlung führen, durch Stoffe, die differnziell den zellulären $cPLA_2$ Verkehr kontrollieren.

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Pectora roborant cultus recti

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Units of measurement

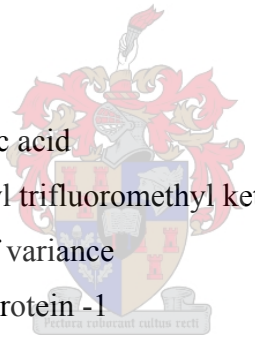
A	ampere
%	percentage
ºC	degrees Celsius
µl	microlitre
µg	microgram
µm	micrometer
µM	micromolar
g	gram
hrs	hours
kDa	kilodalton
l/L	litre
M	molar
Mg	milligram
min	minute
ml	millilitres
mM	millimolar
nm	nanometre
V	volt
U	units

Chemical compounds

APS	ammonium persulfate
BSA	bovine serum albumine
CO_2	carbon dioxide
DMEM	Dulbecco´s modified eagle´s medium
2DG	2-deoxy-D-glucose
FCS	Fetal calf serum
H_2O	water

HS	horse serum
KCl	potassium chloride
$MgSO_4$	magnesium sulphate
NaCl	sodium chloride
O_2	oxygen
PBS	phosphate buffered solution
PenStrep	Penicilin Streptomycin
PVDF	poly vinylidene fluoride
PMFS	Phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis
TBST	Tris-buffered saline-0.1% Tween 20
Temed	N,N,N,N'-Tetramethylethylendiamin

Other abbreviations



AA	arachidonic acid
$AACOCF_3$	arachidonyl trifluoromethyl ketone
ANOVA	analysis of variance
AP-1	activated protein -1
ATP	Adenosine triphosphate
Bp	base pairs
$cPLA_2$	cytosolic phospholipase A_2
cGMP	cyclic 3´, 5´-guanosine monophosphate
COX	cyclooxygenase
DNA	deoxyribonucleic acid
DD	death domain
ER	endoplasmic reticulum
iNOS	inducible nitric oxide synthase
$iPLA_2$	Ca^{2+} independent phospholipase A_2
ERK	extracellular-regulated kinases
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
HEK	human embryonic kidney

HSP	heat shock protein
ICAM	intercellular adhesion molecule
Inh	inhibitor
IPC	ischaemic preconditioning
I/R	ischaemia and reperfusion
JNK	c-Jun NH_2-terminal kinase
K_{ATP} channel	ATP dependent potassium channel
LOX	lipoxygenase
MADD	MAPK activating death domain
MAPK	mitogen-activated protein kinase
MAPKAP	MAPK activated protein
MKK	MAPK kinase
MMP's	matrix metallo proteinases
MKKK	MAPK kinase kinase
NYHA	New York heart association
n	number of experiments
NFkB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthase
PAF	platelet-activating factor
PARP	poly (ADP-ribose) polymerase
PC	preconditioning
PLA_2	phospholipase A_2
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinsase
RIP	receptor-interacting protein
RTK	receptor tyrosine kinase
ROI	region of interest
ROS	reactive oxygen species
SAPK	stress activated protein kinase
$sPLA_2$	secretory phospholipase A_2
SRF	serum response factor
SI	simulated ischaemia

SWOP	second window of protection
TACE	TNF-α converting enzyme
Tex-red	Texas red
TNF-α	tumour necrosis factor alpha
TNFR	tumour necrosis factor alpha receptor
TNF-α PC	tumour necrosis factor alpha preconditioning
TRAF	TNF-α receptor associated factor
UV	ultra violet
Z-DEVD-FMK	Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl Ketone
3-D	3 dimensional

Motivation

There is increasing evidence that inflammatory reactions in infarcted tissue have a major impact on the outcome of the infarction *per se* (Pasupathy and Homer-Vanniasinkam, 2005). The consequences of those reactions, which are of local and systemic nature induce a positive feedback mechanism which worsens the injury over time (Mann, 1996). However, many tissues have the inherent capacity to increase their resistance to an ischaemic insult and to decrease the generation of inflammatory mediators. This phenomenon, where a short ischaemic period prior to a subsequent prolonged ischaemic event reduces the extent of infarction, was termed ischaemic preconditioning (IPC). When this powerful anti-infarct effect was discovered in 1986 (Murry *et al.*, 1986), great hope arose for the development of effective treatment for cardiac illness. However, despite advances in the prevention and treatment of heart disease, cardiac illness still represents a growing threat to our society (Bradshaw, 2005). Not only is there no satisfactory means to treat heart disease but heart failure is also a progressive disease, continuously reducing the patient's quality of life.

The cytokine tumour necrosis factor alpha (TNF-α) has been the centre of attention in this context. Its effects vary according to the dose: at low doses it has been shown to mimic the effect of preconditioning (Lecour *et al.*, 2002), whereas higher doses have a negative effect on heart function, with the levels of TNF-α in the heart being proportional to the degree of heart failure (Seta *et al.*, 1996). However, very recent clinical trials with the aim of reducing this cytokine concentration in heart failure patients, had to be stopped due to the disappointing outcome. These findings call for more in-depth understanding with regard to the signalling mechanisms involved (Mann, 2005). One important role player influencing inflammation in infarcted tissue is the inflammatory mediator generating enzyme cytosolic phospholipase A_2 ($cPLA_2$), which, since it is calcium dependent, takes a special place within the ischaemic context. We therefore became interested in the role of $cPLA_2$ in the cytoprotective effect associated with low dose TNF-α. We hypothesize a role for $cPLA_2$ in TNF-α mediated cytoprotection, possibly on the level of $cPLA_2$ activation and phosphorylation, or through a specific $cPLA_2$ translocation. This motivated us for the conduction of this study.

We hope that our results widen our ideas and indicate new doors for the development of pharmacological agents which can bring about an effective anti-infarct therapy and enable us to optimize our knowledge applied in clinical trials.

Chapter 1: Literature Review

1. Preconditioning

1.1. Concept and context of ischaemic preconditioning

1.1.1. Introduction

In 1986 a powerful anti-infarct effect was discovered. It was found that exposure of the myocardium to a brief episode of sub-lethal ischaemia and reperfusion markedly reduced tissue necrosis induced by subsequent prolonged ischaemia (Murry *et al.*, 1986). This phenomenon was termed ischaemic preconditioning (IPC).

The term *ischaemia* describes "the hindrance of blood supply and the increase in the resistance of flux" and is derived from the Greek "ischein", to restrict and "haima", meaning blood. The term *clinical ischaemia* has since been refined to mean "a relative shortage of blood supply in the coronary circulation, which induces a shortage in the supply of oxygen and substrate and leads to the accumulation of metabolites". This condition of ischaemia can be mimicked in an experimental setup and is termed *simulated* or *experimental ischaemia*. It involves aerobic/anaerobic metabolic inhibition of tissue as well as a low pH, a high potassium concentration and hypoxia. The term *index ischaemia* is used to describe the prolonged ischaemic event.

Twenty years after the discovery of IPC, this endogenous myocardial protection is still a field of intense research. Despite advances in the prevention and treatment of heart disease, cardiac illness ranks as the most frequent cause of mortality. In the United States it has reached epidemic proportions (Cohen *et al.*, 2000). The South African National Burden of Disease study for the year 2000 estimated that 17% of all deaths were due to cardiovascular diseases, where ischaemic heart disease accounted for 35% of cardiovascular deaths (Bradshaw, 2005). According to the World Health Organization cardiac illness will be the major cause of death in the world as a whole by the year 2020 (Murray and Lopez, 1997).

1.1.2. Reperfusion injury

Prolonged occlusion of a coronary artery can have deadly effects on the myocardium it supplies. Because infarct tissue is no longer able to contract, the global function of the heart can be severely impaired. Restoration of the circulation brings about reperfusion of the cardiac tissue and is essential for the re-establishment of oxidative phosphorylation and removal of accumulated waste products. However, reperfusion induces tissue damage, termed *reperfusion injury*. This injury has two serious consequences, of local and of systemic nature. Whilst ischaemic injury is mainly caused by tissue oxygen-deprivation and cellular necrosis, reperfusion injury produces an inflammatory response. This inflammatory response feeds back into the local damage, worsens it over time and leads to a systemic insult (Pasupathy and Homer-Vanniasinkam, 2005).

1.1.3. IPC and the current problem of rescuing the myocardium

Although the concept of IPC describes one of the most powerful methods of minimising ischaemia/reperfusion (IR) injury, currently the only proven way of rescuing the ischaemic myocardium is urgent revascularization of the involved coronary artery, with the aim of minimizing both ischaemic and reperfusion injury. This can be achieved with pharmacological agents to lyse the occluding thrombus, with catheter-based interventions and angioplasty, with stenting or atherectomy. These techniques are all intended to either mechanically displace or remove the occluding atherosclerotic plaque. Alternatively, surgical bypass procedures may be used, to re-route arterial blood around the obstruction (Cohen *et al.*, 2000). However, even if these sophisticated methods are available, the period between patient presentation and successful revascularization is often too long to avoid considerable infarction and reperfusion injury. This time period is crucial since the extent of tissue necrosis is proportional to the duration of the time interval between coronary occlusion and re-established flow (Cohen *et al.*, 2000). It is therefore desirable to make the tissue less susceptible to ischaemic and reperfusion injury and to develop a means of protecting the heart from infarction before revascularization takes place.

IPC has the potential to play a main role in fulfilling these goals. So far our knowledge certainly underlines the complexity of IPC. It has been argued that we know enough to translate accumulated data into effective clinical application (Opie, 1997). On the other hand, disappointing clinical trials especially regarding inflammatory role players demand more in-depth knowledge. It is hoped that elucidation of the mechanism for IPC will yield therapeutic strategies capable of not only reducing the severity of myocardial infarction but also reducing the inflammatory response, which is induced following cardiac tissue necrosis. Therefore the centre of attention is the pathway or pathways used for IPC and the manipulation of the signalling events defining the pathway, which can lead to the development of effective anti-infarct therapy.

1.2. Ischaemic Preconditioning

1.2.1. Infarct size limitation is possible in many species, including humans

The report of a delay in lethal cell injury in myocardium due to ischaemic preconditioning put the scientific community on alert. For the first time it was shown that infarct size limitation was possible. It was demonstrated that four 5 min brief cycles of I/R via occlusion of the coronary artery provided robust myocardial protection from a further prolonged 40 min ischaemic insult in the canine heart. The zone of infarcted myocardium from these hearts was reduced by 70% compared to control hearts at 4 days post-reperfusion (Murry *et al.*, 1986). Similar and reproducible results were observed in other species including rats (Mitchell *et al.*, 1995), rabbits (Cohen *et al.*, 1991), chicken, mouse, sheep and pig (Liang and Gross, 1999). For ethical reasons it has not been possible to test directly whether preconditioning can protect human hearts against infarction, but evidence speaks for it. It has been demonstrated that recovery of function in isolated human atrial trabeculae, after an extended period of hypoxia, was greatly enhanced by earlier hypoxic preconditioning. Furthermore, preconditioning has been shown to protect human ventricular myocytes against simulated ischaemia *in vitro* (Cohen *et al.*, 2000).

1.2.2. Time frames and dose response dynamics of IPC

Two different time frames are defined for IPC, one described as early or classical preconditioning and one as late, delayed or the so called second window of preconditioning (SWOP) (Kuzuya *et al.*, 1993). Early preconditioning provides protection for about 2-3 hrs, whilst SWOP remains protective for 48-96 hrs (Bolli, 2000) (Figure.1.1.). The magnitude of protection differs however, as well as the mechanism leading to protection (Pasupathy and Homer-Vanniasinkam, 2005). It is thought that immediate protection is due to the activation of membrane receptors and post-translation events, whilst delayed protection more likely involves gene expression and protein synthesis, such as the overexpression of heat shock proteins (Perrault and Menasche, 1997; Bolli, 2000). Regarding the dose response dynamics of IPC, conflicting information is available. On the one hand it has been proposed, that it is highly likely that repeated preconditioning episodes are neither additive nor cumulative (Pasupathy and Homer-Vanniasinkam, 2005). However, it has been shown that there is a difference in the degree of protection depending on single-or multi-cycle ischaemia (Sandhu *et al.*, 1997). A very steep dose-response curve for IPC has been described (Pasupathy and Homer-Vanniasinkam, 2005). Once a maximal response is achieved, further stimulation has no additional effect (Pasupathy and Homer-Vanniasinkam, 2005). It is further of note that preconditioning´s protection is lost when the ischaemic insult is extended to 3 hrs, indicating that reperfusion after a lethal ischaemic insult is an absolute necessity for protection (Yellon and Downey, 2003). The duration of the reperfusion period after the preconditioning stimulus has to be at least 30-60 seconds, in order to provide protection (Alkhulaifi *et al.*, 1993). IPC-induced protection is manifested through delaying cellular death rather than preventing it. A time delay reducing the actual duration of the ischaemic insult by 20 to 30 min has been suggested (Yellon and Downey, 2003). Therefore, preconditioning increases the tissue's capacity for tolerating an ischaemic insult and thereby *buys out lethal time* for the benefit of tissue survival.

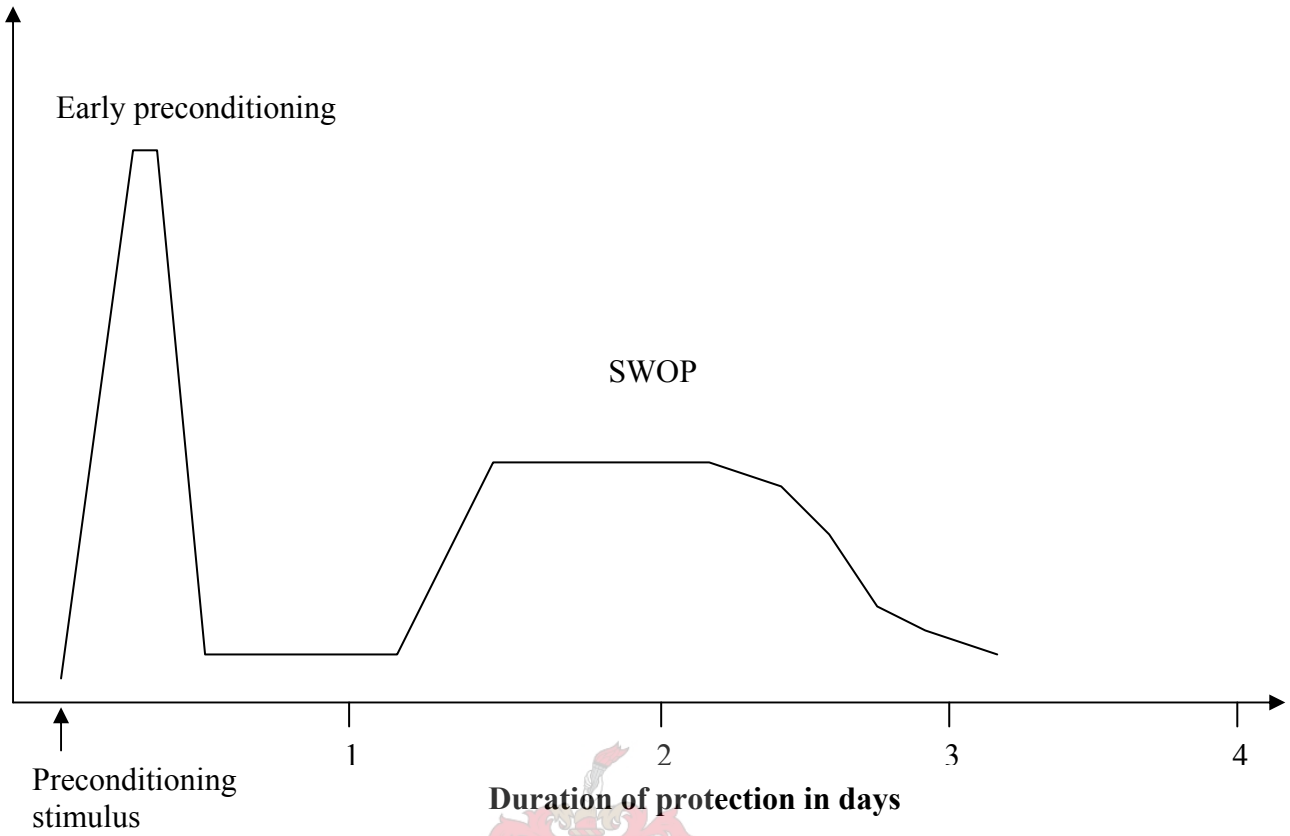


Figure 1.1. Biphasic protection induced by a preconditioning episode (modified from Pasupathy and Homer-Vanniasinkam, 2005).

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1.2.3. Physiological Mechanisms of I/R tolerance

The following physiological mechanisms by which ischaemic preconditioning induces ischaemia tolerance, have been suggested (Pasupathy and Homer-Vanniasinkam, 2005).

- Ionic equilibrium during prolonged ischaemia is maintained
- Cellular energy demand is reduced
- Cellular ATP consumption is reduced
- Rate of glycolysis is reduced
- Intra-cellular energy relays become more efficient
- H^+ production is decreased due to a better maintained ionic household linked to the less affected Na/K ATPase

Many of these mechanisms in the ischaemic phase are directly or indirectly related to the cellular energy household (Hearse, 1994). On the other hand, the parameters whereby preconditioning induces reperfusion tolerance, are centred around a better maintained intracellular redox potential, a reduced production of free oxygen radicals, a reduction in the release of cytokines as well as the preservation of mitochondrial integrity (Pasupathy and Homer-Vanniasinkam, 2005).

1.3. Cellular Mechanisms of IPC and Pharmacological Preconditioning

Most of the insight into the pathway mechanisms of IPC has been established in cardiac muscle tissue compared with skeletal muscle tissue. It has been shown that IPC is indeed a phenomenon of the myocardial cell (Piper and Ladilov, 1997) and an immense body of knowledge regarding the signalling pathways has been established through using cardiomyocytes isolated from the adult or neonatal rat heart. The isolated cell model has shown that when submitting cardiomyocytes to a pharmacological preconditioning protocol through protein kinase C (PKC) stimulation, the cell is protected against Ca^{2+} overload in the ischaemic event. Therefore preconditioning could be thought of as a lessening of calcium-mediated damage (Opie, 1997). Under reoxygenation conditions, cardiomyocytes have been

shown to be protected against the occurrence of reoxygenation induced hypercontracture (Piper and Ladilov, 1997). How the tissue remembers, mechanistically, that it has been preconditioned, seems to remain a mystery. Gene expression can most likely be eliminated since when protein synthesis is inhibited, the early preconditioning´s protection cannot be blocked (Thornton *et al.*, 1990). Furthermore, a preconditioned state can be achieved within 10 min, leaving an unrealistic time window for protein expression. However, reversible posttranslational protein modifications, such as phosphorylation, dephosphorylation and translocation seem much more likely events that bring about the cellular response to IPC.

1.3.1. The Preconditioning pathway - an interplay in series

The mechanism of IPC is emerging as a complex interplay of receptors, protein kinases, and ion channels in series (Cohen *et al.*, 2000). Locally released agonists, such as adenosine, bradykinin, catecholamines and opioids trigger the protection via cell surface G-protein coupled receptors (Pasupathy and Homer-Vanniasinkam, 2005). Therefore, IPC is a receptor mediated phenomenon with a highly redundant receptor system. Kinases like PKC, protein tyrosine kinase (PTK) and p38 mitogen activated protein kinases (MAPK) participate in the signalling pathway. The end-effector remains elusive, although the mitochondrial ATP-sensitive potassium (K_{ATP}) channel might likely serve this role, preventing the opening of the mitochondrial permeability transition pore (Cohen *et al.*, 2000), (Yellon and Downey, 2003) (Pasupathy and Homer-Vanniasinkam, 2005) (Figure 1.2.). Many studies indicate that drugs can imitate or prevent IPC. Due to the cascade-like fashion of the pathway, there are multiple points offering intervention, which is practically appealing when duplicating the protective action of ischaemically induced preconditioning. Within the signalling pathway of IPC, three levels of molecular targets can be considered: triggers, mediators and effectors (Perrault and Menasche, 1997). Triggers act before simulated ischaemia whilst mediators act during the simulated ischaemic event. This classification however should be seen as a guiding framework only. Although literature often refers to these terms, their application demands caution, since borders often overlap resulting in grey areas.

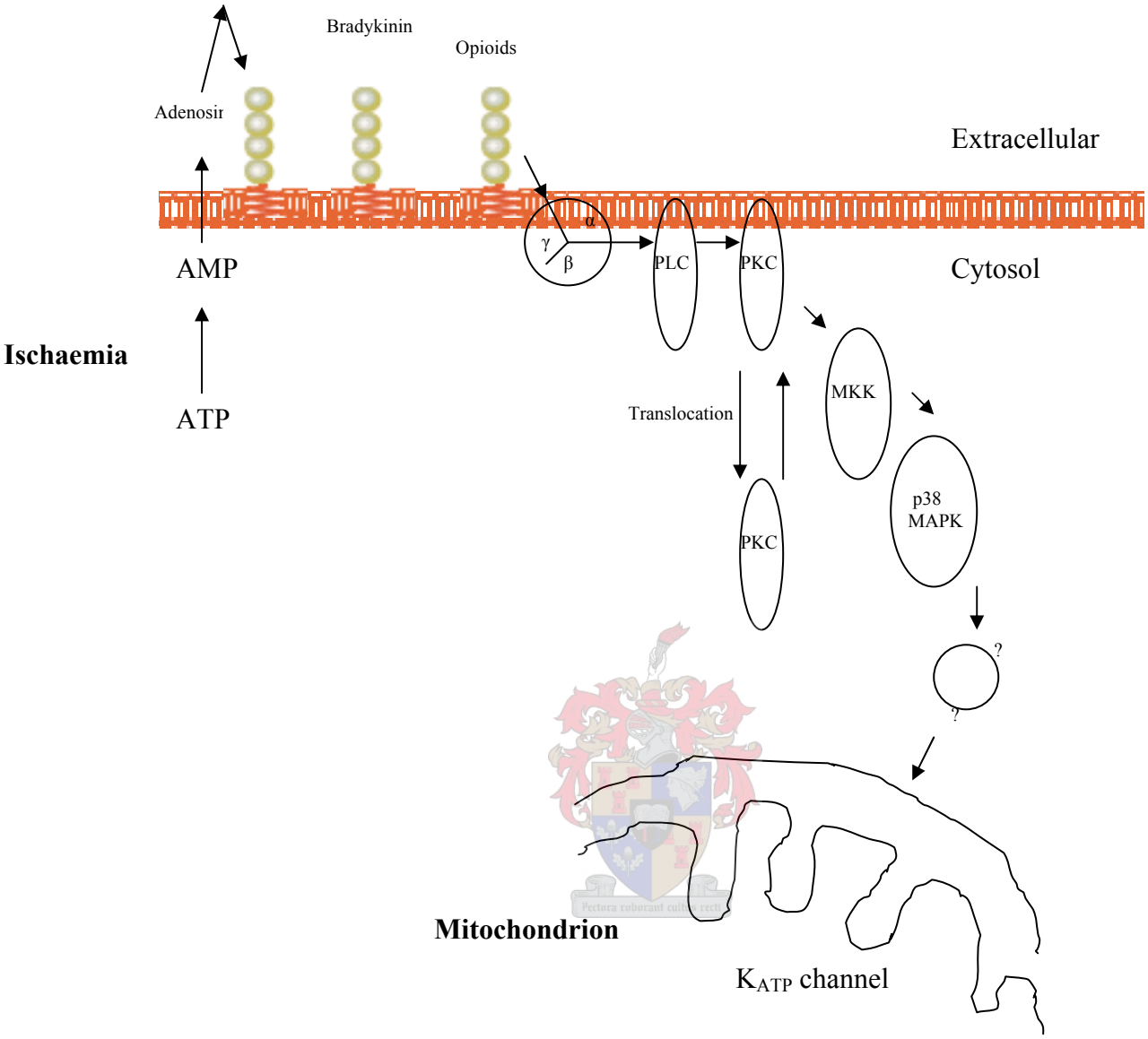


Figure 1.2. A simplified schematic representation of a proposed mechanism of IPC is shown. Ischaemia leads to the release of adenosine, bradykinin and opioids that can activate phospholipase C (PLC); PLC can lead to the translocation and activation of protein kinase C (PKC) which can initiate a complex kinase cascade involving mitogen activated protein kinases (MAPKs). This can lead to the opening of mitochondrial K_{ATP} channels. However, many steps within this sequence remain unknown (modified from Cohen *et al.*, 2000).

1.4. Skeletal Muscle Preconditioning

Several investigators have shown that ischaemic preconditioning also serves to protect skeletal muscle tissue from subsequent ischaemic damage. It is likely that molecular mechanisms of preconditioning in skeletal muscle are virtually identical to those in myocardial tissue (Kohin *et al.*, 2001). This has been demonstrated in the whole organ, tissue and cellular level (Pang *et al.*, 1997). Most work in the field has been done by Pang and colleagues, working with porcine skeletal muscle. He and his group speculated for the first time that there is potential clinical application of the ischaemic preconditioning phenomenon for skeletal muscle against infarction if the protective effect can be induced in the same way as in cardiac muscle (Pang *et al.*, 1995). They observed a 44% and a 62% decrease in the extent of muscle infarction in latissimus dorsi and gracilis muscle flaps respectively, when these muscles were subjected to 4 hrs of ischaemia and 48 hrs of reperfusion. Furthermore, preconditioning was associated with higher muscle content of high energy phosphates and less muscle accumulation of lactate during sustained ischaemia compared with the control muscle flaps (Pang *et al.*, 1995). Subsequently more effort was spent on determining the optimal ischaemic time and number of cycles, to apply IPC most effectively (Saita *et al.*, 2002). It was shown that three to five cycles of 10 min preconditioning achieved a superior protective effect compared with one or two cycles (Saita *et al.*, 2002). Preconditioning of single skeletal muscle fibres also improved function and recovery during severe hypoxia and reoxygenation. In this experimental setting, hypoxia alone was used to ischaemically challenge the cells, without modulating the cellular environment in terms of pH, buffer and metabolic inhibition. An improved force production and attenuation of changes in Ca^{2+} handling was observed (Kohin *et al.*, 2001). Homer and colleagues have demonstrated in a rodent hindlimb model after 6 hrs of ischaemia and 4 hrs of reperfusion, that muscle blood flow during the revascularisation period was reflected in a triphasic pattern, low reflow at 10 min of reperfusion, improving at 120 min, and significantly diminished blood flow at 240 min of reperfusion, representing the reperfusion injury (Homer-Vanniasinkam *et al.*, 2001).

In skeletal muscle, the deleterious effects of I/R appear to be closely associated with proinflammatory processes. There is experimental evidence indicating the possibility

that preconditioning may reduce platelet aggregation and neutrophil adherence in ischaemic skeletal muscle (Pang *et al.*, 1995). Increased intracellular Ca^{2+} during I/R seems to be a primary cause of post ischaemic injury in muscle cells. Ca^{2+} homeostasis may initially be disrupted through reactive oxygen species (ROS) induced injuries and subsequent disturbances in Ca^{2+} handling may follow. Furthermore, elevations in intracellular Ca^{2+} activate proteases and phospholipases that can cause irreversible cellular and mitochondrial damage (Kohin *et al.*, 2001).

1.5. IPC in other tissues

Preconditioning was first described in the heart, but since then it has been described in various organs such as the gut (Ishida *et al.*, 1997) kidney (Bonventre, 2002) and skin (Rosken *et al.*, 1998). Preconditioning in the brain is only protective in the second time window (Kirino, 2002), demonstrating a degree of tissue specificity. However, preconditioning in skeletal muscle seems to be most similar to the preconditioning events found in cardiac tissue (Kohin *et al.*, 2001).

1.6. Clinical Relevance of Preconditioning

There is convincing evidence indicating that ischaemic preconditioning can and will occur in humans. Patients may have experienced numerous angina attacks before suffering from myocardial infarction and some of these episodes may have preconditioned the myocardium. The therapeutic exploitation of these natural adaptive mechanisms in cardiac surgery is intellectually an appealing prospect (Perrault and Menasche, 1997) and has led to successful clinical application of IPC to coronary angioplasty and coronary bypass grafting (Pasupathy and Homer-Vanniasinkam, 2005). Recent clinical trials in pulmonary and hepatic surgery also involve IPC application (Pasupathy and Homer-Vanniasinkam, 2005). However, clinical progress is very slow. It has been argued that enough knowledge about IPC is available to plan appropriate pharmacological responses (Opie, 1997), and that more experiments would only contribute to more confusion rather than clarification. Others (de Jong and de Jonge, 1997) are convinced that as long as the end effector is elusive, more pharmacological experiments are needed. When looking especially at the differences between low-flow and no-flow induced ischaemic injury, concern has been raised

regarding and suggestions been given, that additional work is needed (de Jong and de Jonge, 1997). Most preconditioning experiments focus on no-flow ischaemic injury after the pre-treatment, however, in the clinical practice low-flow conditions are more likely to occur, due to partial coronary occlusion or collateral flow (de Jong and de Jonge, 1997). The concern addresses differences in receptor binding dynamics due to wash out and agonist availability. Furthermore metabolic and ionic differences are apparent when comparing no-flow and low-flow induced ischaemic injury (El Banani *et al.*, 2000).

Unfortunately, in the case of skeletal muscle preconditioning, clinical progress is also slow. In many clinical conditions, single or multiple skeletal muscle fibres are subjected to global ischaemia and would benefit from IPC. This would be the case during muscle transplantation or surgical muscle reconstruction. The tolerance of skeletal muscle to global ischemia is comparatively large, but only up to a point. When this tolerance is exceeded in excessive ischaemia, I/R injury and muscle necrosis can result. A massive infarction may induce life threatening acidosis and hyperkalaemia. Skeletal muscle reperfusion injury is characterized not only by necrosis but also impaired muscle contraction, endothelial cell swelling, release of intracellular enzymes and proteins and increased microvascular permeability to proteins (Homer-Vanniasinkam *et al.*, 2001). In diabetic and nondiabetic patients with disturbed haemodynamic parameters such as in lower limb arterial disease, defined by recurring ischaemic rest pain, IPC might be an additional treatment strategy for optimal medical care (Vayssariat *et al.*, 1997). Following surgery for acute limb ischaemia, a major amputation may be required in up to 15% of cases despite a technically successful operation (Homer-Vanniasinkam *et al.*, 2001).

In summary, knowledge and understanding of the mechanism employed in IPC demands translation to the bedside. It seems that the application of what we do know is overridden by the wish to increase our understanding of existing data and the fear of inducing side effects such as arrhythmias when for example intervening with cardiac ATP-sensitive K^+ channel modulating drugs (Opie, 1997).

2. TNF-α

2.1. TNF-α - the proinflammatory cytokine in myocardial homeostasis

The functional role of Tumor necrosis factor-alpha (TNF-α) in the myocardium and other tissues has been studied extensively over the past several years. However the partly beneficial as well as potentially adverse effects of TNF-α in the cardiovascular setting are only marginally understood. With the "cytokine hypothesis" 10 years ago, it was recognized that the pro-inflammatory cytokine, TNF-α, is involved in virtually all forms of cardiac injury. This paradigm shift away from a primarily haemodynamic disorder and towards biologically active molecules such as cytokines has since been substantiated. The term *cytokine* describes a group of small molecular weight proteins (15-30 kDa), that are secreted by a broad variety of cells in response to different inducing stimuli. In response to cellular environmental stress, they act primarily in a juxtacrine, paracrine and autocrine fashion; however when produced in large amounts, they also can exert endocrine effects.

Two major classes of cytokines have been identified, vasoconstrictor cytokines such as endothelin and "proinflammatory" cytokines such as TNF-α (Seta *et al.*, 1996). Despite recruiting and stimulating cellular components of the immune system, proinflammatory cytokines induce their own production as well as the synthesis of small inflammatory mediators such as platelet-activating factor (PAF) and oxidative radicals (Meldrum, 1998). TNF-α was originally discovered as a necrosis-inducing protein in certain mouse tumours. Now it is recognized as a cytokine with pleiotropic capacities, including effects on growth, differentiation and apoptosis in virtually every cell type investigated, including cardiac myocytes and skeletal muscle myotubes (Li and Schwartz, 2001). The pleiotropic effect of TNF-α is facilitated via the activation of caspases, mitogen activated protein kinases (MAPKs), transcription factors, activated protein-1 (AP-1) and nuclear factor-kB (NF-kB). In this way TNF-α mediates pro-apoptotic and pro-survival mechanisms in the cell. It has been suggested that TNF-α adjusts the redox potential of the cell through reactive oxygen intermediates, which mediate parts of the decision making of the cell between survival or apoptotic death (Garg and Aggarwal, 2002). Furthermore, it is part of a

network that orchestrates inflammatory events. TNF-α and TNF-β share similar inflammatory activities; however, TNF-β, or as it was first described, "Lymphotoxin" is less potent and mainly produced by T-cells, whereas macrophages are the predominant generators of TNF-α.

Confirmed in numerous experimental materials, it has been shown that TNF-α plays an important role in mediating and integrating the myocardial response to stress (Mann, 1996). It plays a crucial role in maintaining myocardial homeostasis via the integration of cytoprotective-, hypertrophic- or repair-orientated responses (Mann, 2003). According to the cytokine hypothesis, heart failure is induced because cytokine cascades intensify haemodynamic abnormalities or exert direct toxic effects on the tissue (Seta *et al.*, 1996).

2.2. TNF-α synthesis and control

The various control mechanisms from gene induction to post-release buffering underpin the potency of TNF-α. Although TNF-α generation was initially described as a lipopolisaccharide (LPS) induced macrophage product, it has now been shown that cardiac myocytes themselves are capable of substantial TNF-α synthesis, when stimulated appropriately (Meldrum, 1998). In fact, the myocardium is capable of generating amounts of TNF-α equal to those in the liver or spleen, organs with large macrophage populations (Meldrum, 1998). After an ischaemic event, TNF-α accumulation can be visualised immunohistochemically within the cardiomyocytes, while before ischaemia, only cardiac residential macrophages show a TNF-α signal. TNF-α gene expression is tightly regulated and baseline levels are rapidly restored after the stress stimulus has ceased. Nuclear factor kappa B (NFκB), a redox-sensitive transcription factor, regulates a battery of inflammatory genes, amongst them the TNF-α gene (Valen *et al.*, 2001). Once NFκB is liberated from the inhibitory protein IκB, it translocates from the cytoplasm to the nucleus, where it docks to one of four TNF-α promoter sites. Once the TNF-α gene has been transcribed, TNF mRNA is translated into a 26-kDa TNF precursor or a 233 amino acid pro-hormone. A transcriptional control-, as well as post transcriptional stabilisation mechanism of TNF-α mRNA has been suggested to govern TNF-α availability (Mann, 1996). Because TNF-α contains a 33 nucleotide 3´-untranslated sequence that shortens

messenger RNA half life, the production of large quantities of this potent peptide is inhibited (Feldman *et al.*, 2000). The mature 17-kDa and 157 residue TNF-α is released into the extracellular space after cleavage by a TNF-α converting enzyme (TACE) (Feldman *et al.*, 2000). TNF-α mRNA is not detectable in naive hearts, but is expressed *de novo* within 30 minutes following a stressful event (Mann, 1996). The same pattern is followed for biologically active TNF-α protein. In the presence of noxious stimulants including TNF-α and LPS, TNF-α receptors are "shed" into the circulation as truncated fragments of the extracellular regions (Feldman *et al.*, 2000). This mechanism serves as an additional post-release control to biologically "buffer" TNF-α activity.

Due to the extensive study of macrophages, the intracellular signalling pathways that provoke TNF-α production have been elucidated. Lipopolysaccharides (LPS) or ischaemia/reperfusion activate myocardial p38 MAPK and NFκB, which leads to TNF-α production. Binding of LPS binding protein (LBP) to CD 14 surface receptors triggers TNF-α production (Meldrum, 1998). This protein receptor interaction also causes rapid activation of protein tyrosine kinase, which in turn induces phosphorylation of intracellular protein kinases such as MAP kinase kinase. Ras is an early target of activated PTK and interacts directly with Raf-1. Raf-1 is an important intermediate to MAPK activation. The p38 MAPK appears to be a pivotal MAPK in the cascade leading to TNF-α gene induction (Meldrum, 1998).

2.3. TNF-α pathways

2.3.1. TNF-α receptors and downstream pathways

The K_d of TNF-α for its receptor was found to be in the range of 0,3 to 1 nM, indicating the TNF-α level required for eliciting a physiological effect (McVey *et al.*, 1999). Biological effects of TNF-α are induced by binding to two distinct cell surface receptors, known as TNFR1 and TNFR2, with a molecular weight of 55 kDa and 75 kDa respectively. They signal as homotrimers and can exist either membrane-bound or as a truncated soluble form (Sack, 2002). The presence of both receptors in the heart has been described and their location has been immunolocalized. Although they share homology in their extracellular domains, the intracellular parts are different and

suggest distinct modi for signalling and cellular function. Signalling pathways coupled to the high affinity TNFR1 are known in more detail and include the activation of phospholipase A_2, phospatidylcholine specific phospholipase C and activation of neutral and acidic sphingomyelinases. TNF-α induces sphingosine mediated apoptosis via TNFR 1 and its "death domain", whilst NFκB is induced via TNFR2. TNFR1 and Fas receptors are linked to cytoplasmic proteins, called TRADD and FADD, TNF receptor associated death domain and Fas-associated death domain respectively (Meldrum, 1998). Communication between these death domain proteins is achieved by receptor-interacting protein (RIP) that facilitates the signal and leads via endonuclease activation to nuclear DNA fragmentation. TNFR2 communicates with TNF receptor associated factors (TRAFs). TRAFs contain zinc finger motifs that enable them to modulate transcription factor activity. To make things more complex, TNFR1 and TNFR2 pathways can cross activate each other and thereby channel the pathway flux (Figure 1.3.).

2.3.2. TNF-α induced apoptosis

Programmed cell death (apoptosis) is a process by which the cell undergoes inducible, ATP dependent, non necrotic cellular suicide. The death domain activation with its subsequent activation of TRADD and FADD and caspase-8, leads to caspase-3 activation and ultimately apoptosis (Garg and Aggarwal, 2002). However, a second pathway mediated via the mitochondria, can be recruited. Reports show that mitochondria are the major source of reactive oxygen intermediates that mediate TNF-α induced signalling (Garg and Aggarwal, 2002). TNF-α alters the mitochondrial membrane permeability, cytochrome c release and subsequent caspase activation, which can lead to apoptosis. Cardiac myocyte apoptosis occurs amongst others in ischaemia and chronic heart failure. The decision making between necrotic and apoptotic cell death is an important process, since it determines the overall organism response dramatically. Apoptosis is characterized by the maintenance of cell membrane integrity, therefore the release of inflammatory mediators such as TNF-α is prevented and a systemic immune response cannot take place. Despite these systemic versus locally controlled consequences, one finds functional implications in this context. For example, apoptotic myocytes, in contrast with their necrotic partners,

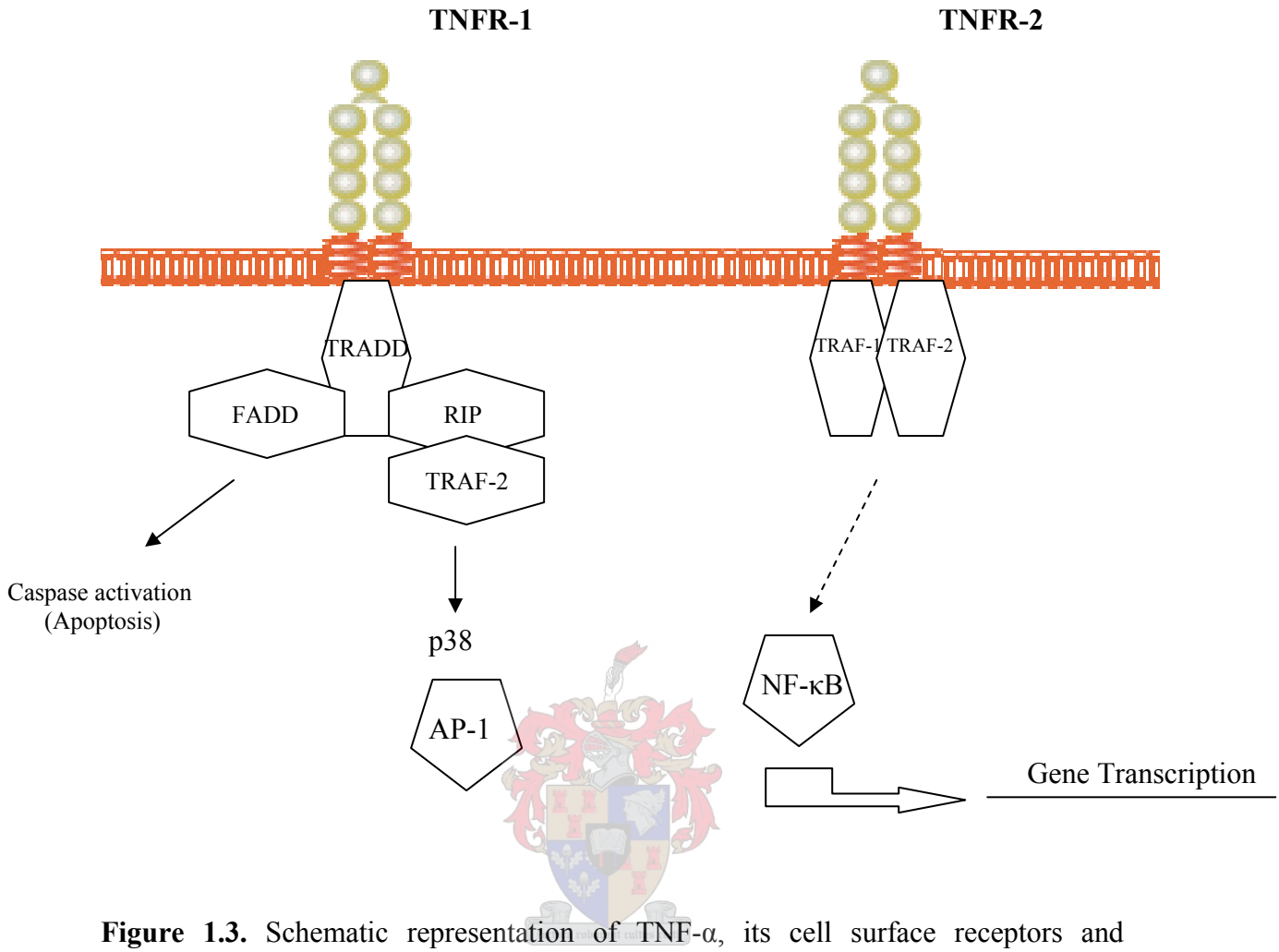


Figure 1.3. Schematic representation of TNF-α, its cell surface receptors and associated signalling molecules. Details are discussed throughout the text. TNFR, TNF-α receptor; TRADD, TNFR-associated protein with death domain; TRAF, TNFR-associated fctor; FADD, Fas-associated death domain; AP-1, activator protein-1; RIP, receptor interacting protein; NFκB, nuclear factor κB (modified from Sack, 2002).

maintain myofilament responsiveness to calcium ionophore, which dramatically determines the outcome of contractile performance (Meldrum, 1998).

2.4. Dose and temporal dependent action pattern of TNF-α

The TNF-α dose and temporal dependent pattern of action is well known and has withstood the test of time (Sack, 2002). It has been shown that the short term versus long term expression of TNF-α brings about a differential response. Short term expression may provide an adaptive response, whereas long-term expression induces maladaptation (Mann, 1996). In 1990 it was recognized for the first time, that circulating levels of TNF-α were elevated in patients with end stage heart failure and cachexia (Feldman *et al.*, 2000). Elevated levels of TNF-α are detected peripherally following myocardial infarction as well as in unstable angina, myocardial reperfusion injury, hypertrophic cardiomyopathy and heart failure (Mann, 1996). TNF-α has also been recognized as a mediator responsible for the contractile dysfunction following coronary microembolization, a frequent complication of atherosclerotic plaque rupture (Dorge *et al.*, 2002).

2.4.1. TNF-α overexpression

Cardiac specific overexpression of TNF-α in transgenic mice has shown that robust amounts of expression have a lethal outcome due to fulminant myocarditis. In transgenic mice where TNF-α expression levels allow the development of viable mice, they present with a similar pathologic, cellular and molecular phenotype to that seen in heart failure in humans, eg. chamber dilation, myocyte hypertrophy and matrix remodelling with fibrosis (Feldman *et al.*, 2000).

2.4.2. Maladaptive response

Maladaptive responses due to a high dose TNF-α have been suggested to lead to left ventricular dysfunction, remodelling and cardiomyopathy as subsequent consequences. Direct injections of TNF-α produce hypotension, metabolic acidosis and death within minutes, mimicking the cardiac response seen during endotoxin

induced septic shock. Myocardial contractility is gradiently affected where an increasing TNF-α concentration leads to decreased myocardial contractility, an effect which is reversible upon removal of the cytokine (Mann, 1996). This is confirming the TNF-α induced production of immediate and delayed negative inotropic effects. Despite decreased contractility, haemodynamic effects of TNF-α are seen in a reduced ejection fraction, a decreased systemic vascular resistance and biventricular dilation (Meldrum, 1998).

2.4.2.1. Mechanism of maladaptive responses

Because calcium handling is crucial in facilitating contraction-relaxation, this field has been of special interest regarding maladaptation due to TNF-α. A decrease in the systolic amplitude of the calcium transient was observed after TNF-α application (Meldrum, 1998). This transition, a calcium induced calcium release from the sarcoplasmic reticulum ryanodine receptor is disturbed due to a disruption in the sarcolemmal voltage gated L-type calcium channel induced calcium influx (Meldrum, 1998).

The biphasic maladaptive response of the myocardium suggests two different mechanisms. Sphingosine and NO have been described as two players which can modulate the calcium transient (Meldrum, 1998). Sphingosine on the one hand might mediate the disrupted calcium release. It is rapidly produced and blocks the ryanodine receptor, thereby driving the calcium dyshomeostasis. Blockage of sphingosine production abolishes TNF-α induced contractile dysfunction. NO on the other hand mediates TNF-α induced desensitization of myofilaments to intracellular calcium (Meldrum, 1998). TNF-α is thought to increase the expression of the inducible nitric oxide synthase (iNOS). NOS inhibition could prevent the TNF-α induced myocardial depression (Meldrum, 1998). Also, a causally involved signal transduction cascade of NO, TNF-α and sphingosine has been described in coronary microembolization-induced contractile dysfunction (Thielmann *et al.*, 2002).

Other contributors to the maladaptive response are direct TNF-α induced cytotoxicity, oxidant stress, myocyte apoptosis and the induction of other cardiac depressants such as interleukins (Meldrum, 1998). Furthermore, since TNF-α activates matrix

metalloproteinases (MMPs), changes in the balance between MMPs and tissue inhibitors of MMPs (TIMPs) activity are induced (Sarzi-Puttini *et al.*, 2005). This could influence intercellular matrix composition, collagen turnover and flux of signalling molecules.

2.4.3. Adaptive responses

A growing body of evidence supports the notion that short term expression of pro-inflammatory cytokines is beneficial. TNF-α has also been shown to up-regulate the expression of heat shock protein 72 (HSP 72), a protein thought to protect the heart against injury (Mann, 2003). It has been observed, that a concentration of < 10 U/ml TNF-α has no effect on HSP 72 expression, while a concentration of 50 U/ml produces significant increases. Although 1 unit (U) describes the amount of enzyme that catalyzes the reaction of 1 μM of substrate per minute, it is seen here as TNF-α activity. Pathophysiological TNF-α levels, such as 1000 U/ml result in less HSP 72 levels compared to a 200 U/ml dose. Thus, short term TNF-α expression may provide additional homeostatic responses such as increased regional blood flow, increased resistance to ischaemia-induced arrhythmias through nitric oxide generation, increased free radical scavenging through increased expression of manganese superoxide dismutase and protection against hypoxic injury (Mann, 1996).

2.4.3.1. Mechanism of adaptive responses and TNF-α preconditioning

The expression of low concentrations of TNF-α for relatively short periods of time provides the heart with a short term adaptive response to stress. These patterns are mirrored when preconditioning the heart with a low dose of TNF-α for a short time period. Despite the capacity of TNF-α to function as an ischaemic preconditioning mimetic, a dose dependent effect of TNF-α determined the outcome of tissue tolerance to ischaemia (Lecour *et al.*, 2002) (Figure 1. 4.). Using recombinant TNF-α as preconditioning stimulus, a dose of 0.5 ng/ml TNF-α was most beneficial in myocardial infarct size reduction following the ischaemic event (Lecour *et al.*, 2002). In this context, a role for sphingolipid second messengers in the TNF-α mediated preconditioning pathway has been suggested, since a ceramidase inhibitor could abolish the cardioprotective effect (Lecour *et al.*, 2002). It has also been demonstrated

that IPC and TNF-α are interlinked via adenosine. It has been shown that adenosine´s anti-inflammatory effects include a decrease in cardiac TNF-α production following I/R injury, as well as the inhibition of neutrophil adhesion to cardiac myocytes (Meldrum, 1998). Ischaemic preconditioning or adenosine stimulation decreased myocardial TNF-α production and improved post-ischaemic functional recovery. These observations suggested a distal effector role of TNF-α production and amplified the attention drawn towards TNF-α as a role player within the preconditioning context (Meldrum *et al.*, 1998). Cardioprotection by ischaemic preconditioning is associated with reduced TNF-α production during I/R (Belosjorow *et al.*, 1999). When preconditioning is performed with TNF-α, it downregulates its own production. Using TNF-α null (TNF-α $^{-/-}$) mice , it was shown that cardiac TNF-α production is a necessity for cardioprotection induced by IPC, substantiating a profound role of TNF-α in the IPC cascade. Furthermore, administration of a low dose recombinant TNF-α mimicked IPC in wild-type mice, but not in the TNF-α deficient species (Smith *et al.*, 2002). Various upstream molecular signalling events, which influence TNF-α production, are present. Other forms of pharmacological preconditioning, such as treatment with calcitonin gene related peptide, are also capable of reducing cardiac TNF-α content (Peng *et al.*, 2000).

2.5. Effects of TNF-α preconditioning on skeletal muscle

A growing body of knowledge has been established regarding the role of TNF-α in skeletal muscle; however more in the context of muscle degradation, myotube differentiation and only lately in TNF-α preconditioning. TNF-α has long been known to mediate skeletal muscle protein catabolism, therefore it was originally known as "cachectin". It appears now, that TNF-α also serves a physiological role in skeletal muscle, since it is generated and up-regulated after strenuous exercise (Li and Schwartz, 2001). It serves also as promoter in early phase myotube differentiation by stimulating NFκB and serum response factor (SRF) activity (Li and Schwartz, 2001). SRF is preferentially expressed in embryonic and adult cardiac and skeletal muscle tissue (Li and Schwartz, 2001). Furthermore, it was shown that TNF-α can provoke a hypertrophic growth response by stimulating muscle proteins like α-actin and myosin heavy chain (MHC) (Li and Schwartz, 2001). Human derived Girardi cells and C2C12 myotubes were both shown to respond to ischaemic and pharmacological

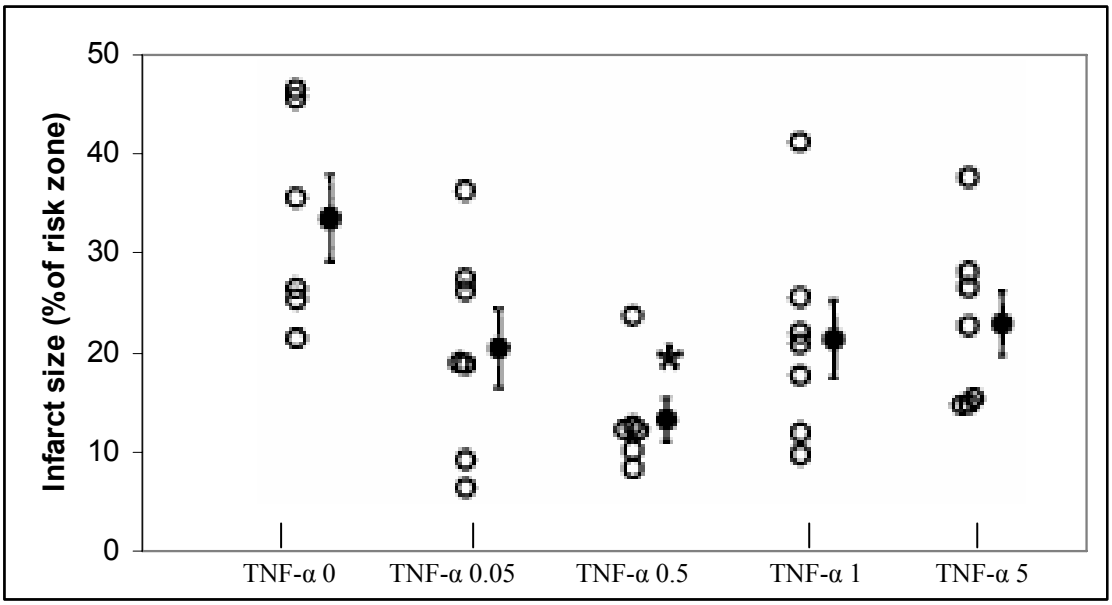


Figure 1.4. The dose dependent cardioprotective effect of TNF-α in ng/ml. Shown is infarct size following 30 min coronary occlusion and 2 hrs of reperfusion. * $p<0.01$ vs conrol group (from Lecour *et al.*, 2002).

preconditioning in a similar manner with the induction of mitochondrial uncoupling. This has drawn the attention to the importance of mitochondrial energetics within the preconditioning programme (Minners *et al.*, 2001). The similarities of skeletal muscle characteristics, compared with cardiac muscle, has led to the application of skeletal muscle cell lines to serve as a model for cardiac research.

2.6. Clinical relevance for anti TNF-α therapy and TNF-α tissue preconditioning

With TNF-α as role player in the clinical context the benefits could be two fold:
1. to decrease TNF-α tissue concentration to decelerate heart failure progression and inflammation and
2. to protect the heart from ischaemic injury.

TNF-α might have prognostic significance in heart failure and as biochemical marker for the progression of left ventricular dysfunction. A progressive increase in peripheral TNF-α levels has been described in direct relation to the patient's New York Heart Association (NYHA) classification in comparison to age-matched control subjects (Figure 1.5.) (Seta *et al.*, 1996). However, for reasons that are unclear, only about 30-40% of the patients with heart failure show this TNF-α elevation.

Anti TNF-α strategies could prove to be beneficial in the treatment of patients with heart failure and a series of phase I clinical trials with moderate to advanced heart failure patients led to promising improvements, such as increased walking distance and ejection fraction (Sarzi-Puttini *et al.*, 2005). Also in subjects with rheumatoid arthritis, treatment with the soluble TNF-α receptor peptide as well as the monoclonal antibody against TNF-α, *infliximab,* has been shown to reduce the symptoms accompanying the disease (Sack, 2002). However, the results following multi-centre trials using the soluble TNF-α receptor peptide, *etanercept,* were very disappointing. In the study a relevant benefit of *etanercept* on the rate of death or hospitalization was ruled out (Mann *et al.*, 2004). Reasons for the disappointing results might lie in a too drastic decrease of TNF-α level below the physiologically relevant levels. Complement fixation, pharmacodynamic interactions and genetic polymorphism as well as race or sex related implications might also have contributed (Sarzi-Puttini *et*

al., 2005). In fact, only recently a study demonstrated gender related differences in the preconditioning threshold as well as a variation in the expression of TNF-α (Pitcher *et al* 2005). If one compares this context with the difficulty of fine-controling blood sugar levels with insulin, one can imagine that this artificial "receptor shedding" is similarly far away from the fine-tuned and tight TNF-α control mechanisms occurring endogenously. A limited immune response such as a limit in the clearance of live bacteria might have been responsible for the mortality in clinical trials (Meldrum, 1998). It has been shown that TNF-α knock out mice are unable to clear bacteraemia after *Listeria monocytogenes* infection. These mice develop normally but are highly susceptible to infections (Meldrum, 1998).

Other ways to decrease cardiac the TNF-α level have to be identified. Inhibition of TNF-α transcription is not feasible, since it is needed in the heart. More distal inhibition, such as p38 MAPK or NFκB inhibition may have a greater appeal. In the mean time physicians are advised to exercise caution when using *eternacept* and not to use TNF- α blockers in patients with NYHA class III or IV (Mann *et al.*, 2004).

3. Cytosolic Phospholipase A_2 ($cPLA_2$)

3.1. $cPLA_2$ and inflammatory mediator synthesis

Phospholipases A_2 (PLA_2) are ubiquitous enzymes that are present in most cells and tissues. Hydrolysis of cellular membranous phospholipids by PLA_2s causes the release of free fatty acids and lysophospholipids. Many of the newly generated lipids have profound biological properties. They promote inflammatory reactions and participate in processes that lead to tissue injury. Four different PLA_2 subfamilies can functionally be distinguished: a secretory PLA_2 ($sPLA_2$), a Ca^{2+} sensitive cytosolic PLA_2 ($cPLA_2$), a Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$) and the PAF acetylhydrolases (PAF-Ahs). The 85 kDa Ca^{2+} sensitive cytosolic PLA_2, also referred to as group IV $cPLA_2\alpha$, has been purified, cloned and biochemically characterized (Kramer and Sharp, 1997). Similar to TNF-α, $cPLA_2$ was first characterized in macrophage cells. It is in fact the best characterized and most relevant isoenzyme for

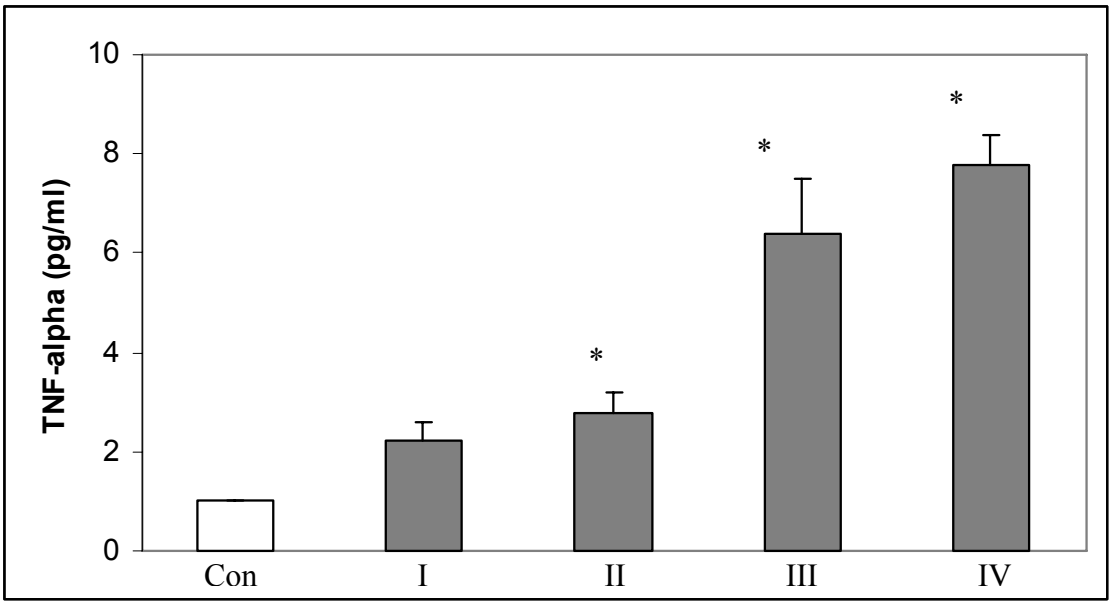


Figure 1.5. TNF-α levels in patients with class I-IV heart failure, in comparison to age-matched control subjects. * $p<0.05$ vs control (modified from Seta *et al.*, 1996).

intracellular signalling because it drives key activities in inflammatory mediator biosynthesis (Dessen, 2000).

Group IV cPLA$_2\alpha$ is the only PLA$_2$ enzyme with marked preference for the second ester bond (sn-2) within the phospholipids molecule, cleaving AA over other fatty acids (Murakami *et al.*, 2003). Arachidonic acid (AA) release is the rate limiting step in the biosynthesis of the potent mediators such as prostaglandins and leukotrienes. Therefore, cPLA$_2$ supplies AA to the downstream cyclooxygenase (COX) and lipoxygenase (LOX) that catalyze the conversion of AA into prostaglandins, leukotrienes, thromboxanes and lipoxins (MacEwan, 1996). Interestingly, the cleavage of phospholipids appears in an order of preference for phosphatidylcholine>phosphatidylethanolamine>phosphatidylserine. The cellular AA levels are controlled by a fine-tuned deacylation-reacylation cycle of phospholipids. In cardiac cells, these processes are governed by lysophospholipid acyl transferase and PLA$_2$. Liberation of AA may occur in a controlled manner, for example via ligand-receptor interactions, or in an uncontrolled fashion. Ischaemia/reperfusion mediates uncontrolled membrane phospholipid hydrolysis, causing significant elevation in AA levels with potential harmful consequences for cellular functioning (Van der Vusse *et al.*, 1997). Additional members of cPLA$_2$, namely cPLA$_2\beta$ and cPLA$_2\gamma$ have been cloned recently (Taketo and Sonoshita, 2002). cPLA$_2$ is ubiquitously expressed in most adult human tissues with most prominent m-RNA levels in brain, heart and lung tissue (Hirabayashi and Shimizu, 2000).

3.2. cPLA$_2$ activation

Two major avenues exist for cPLA$_2$ activation. Firstly, it is the only known PLA$_2$ that is functionally receptor-regulated and therefore takes part in receptor mediated intracellular signal transduction processes (Kramer and Sharp, 1997). Cytokines such as TNF-α can increase the activity of cPLA$_2$ via phosphorylation and the induction of cPLA$_2$ via the p70 TNFR. Secondly, the avenue via non-receptor mediated stimulation is mediated by physical and stressful stimuli such as oxidation, UV light, shear stress and hyperglycaemia. The intensity, duration and cooperative coincidence of the signals determine the immediate activation of cPLA$_2$ (Hirabayashi and Shimizu, 2000). This activation is induced in the presence of 0,3 -2,0 μM Ca^{2+} in the

cytosol and it has been suggested that this Ca^{2+} increase, either derived from an increased influx or from increased mobilisation from intracellular stores, is the predominant factor causing $cPLA_2$ activation (Kramer and Sharp, 1997). A hyperbolic response curve has been described displaying the relationship between enzyme activity and Ca^{2+} concentration (Tong *et al.*, 1998). The Ca^{2+} signalling patterns can occur as single transients, repetitive oscillations or as a sustained plateau and can bring about differential translocation patterns (Hirabayashi and Shimizu, 2000). With a transient [Ca^{2+}] increase and rapid return to basal levels, the translocation is also transient with little AA release. A prolonged [Ca^{2+}] increase over several minutes however, stabilizes the association of the enzyme and its target, resulting in abundant AA release. In this way the cell is able to discriminate between appropriate signals and to prevent "false" activation (Hirabayashi and Shimizu, 2000).

In addition, $cPLA_2$ activity can also be regulated via phosphorylation, resulting in enhanced catalytic activity. Four serine residues were found to serve as phosphorylation sites in human $cPLA_2$, with Ser-505 and Ser-727 conserved in other species. The serine kinases, protein kinase C (PKC) and mitogen activated protein (MAP) kinases, are able to phosphorylate and activate $cPLA_2$. In that context it was shown that $cPLA_2$ is a substrate for p42 as well as p38 MAP kinase via Ser-505 phosphorylation (Figure 1.6). Both MAP kinases capacitate in increasing the intrinsic enzymatic activity of $cPLA_2$ (Kramer and Sharp, 1997). Phosphorylation via MAP kinases on the Ser-505 residue brings about a 2-3 fold increase in activity and it has been suggested that this phosphorylation could optimize substrate accessibility by modulating the inter-domain rotation angle (Dessen, 2000) as described below. Surprisingly however, p38 inhibition did not affect $cPLA_2$ rapid mobilization (Kramer and Sharp, 1997). For maximal activation of $cPLA_2$, phosphorylation has to precede the calcium induced translocation to membranes (Bunt *et al.*, 1997). Importantly, in the absence of an increase in cytosolic Ca^{2+}, phosphorylated $cPLA_2$ fails to induce AA release, since Ca^{2+} is a necessity for $cPLA_2$ binding to the membrane substrate.

Extracellular stimuli can alter the levels of $cPLA_2$ mRNA and protein levels. TNF-α as well as IL-1 and interferon-γ have shown to induce $cPLA_2$ activation and synthesis in diverse cell models (Hirabayashi and Shimizu, 2000). There is also evidence for post transcriptional $cPLA_2$ regulation, since multiple AUUUA sequences in the 3´

region seem to govern mRNA stability (Hirabayashi and Shimizu, 2000). The $cPLA_2$ gene locus also contains the functionally related prostaglandin synthase 2 gene, which would make a coordinated regulation of related enzymes possible (Kramer and Sharp, 1997). In addition, there are consensus sites amongst others for NFκB. The $cPLA_2$ cDNA encodes a 749 amino acid protein with a predicted molecular mass of 85.2 kDa. It is of note that the sequence of murine $cPLA_2$ is more than 95% homologous to the human sequence.

3.3. $cPLA_2$ structure, sub-cellular localization and translocation

$cPLA_2$ preferentially cleaves substrates presented at a membrane interface rather than in monomeric form (Dessen *et al.*, 1999). Analysis of the subcellular distribution of $cPLA_2$ in resting and stimulated cells indicated $cPLA_2$ redistribution from the cytosolic to the membrane fraction upon stimulation. In a variety of cells, a translocation to nuclear and endoplasmic reticulum membranes has been observed, which seems to be cell- and stimulus specific (Kramer and Sharp, 1997). Co-localization studies showed that when endothelial cells are stimulated with a calcium mobilizing agonist, a relocation of $cPLA_2$ was highly specific, primarily to intracellular structures resembling the endoplasmic reticulum and Golgi apparatus as well as to the inner and outer surfaces of the nuclear membrane (Grewal *et al.*, 2005). There is evidence that enzymes such as prostaglandin synthase I and II are localized in these regions, making the translocation locus an ideal position to provide optimal free arachidonic acid supply to the enzymes of the eicosanoid cascade, whose products in turn are subsequently available for intracellular recognition sites (Kramer and Sharp, 1997) (Figure 1.7.). In unstimulated cells a punctuate cytosolic labelling pattern has been observed by electron microscopy: no specific organellar membrane was preferred. This observation, that $cPLA_2$ is localized in clusters, may have important implications for regulation and function (Bunt *et al.*, 1997). The $cPLA_2$ molecule is composed of an N-terminal C2 domain with 138 amino acids and a catalytic unit with 611 amino acids. The C2 domain is responsible for the Ca^{2+} dependent binding of $cPLA_2$ to membranes or phospholipid structures.The Ca^{2+} dependent phospholipid binding domain is essential for the translocation process (Bunt *et al.*, 1997). Even a differential translocation of $cPLA_2$ as a function of $[Ca^{2+}]$

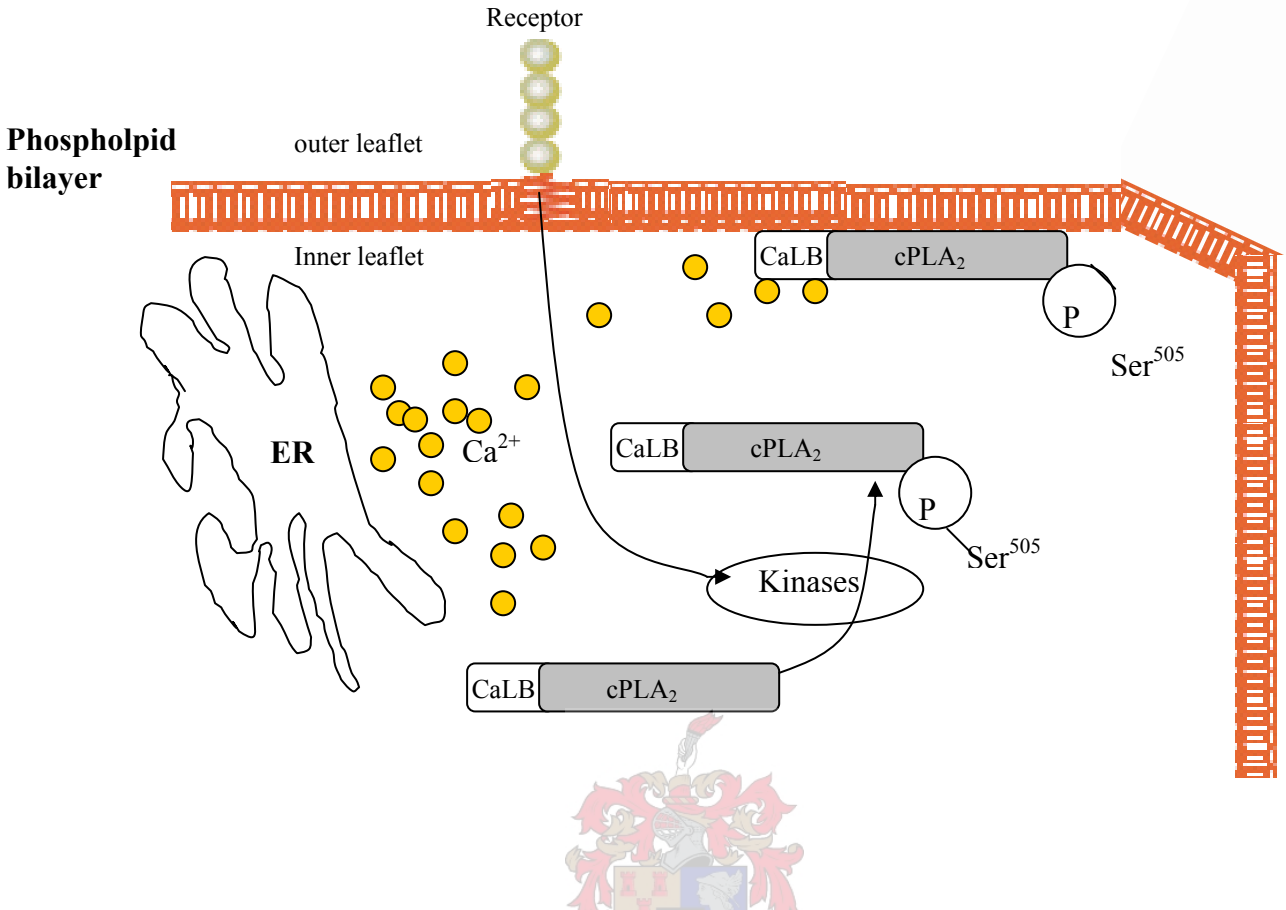


Figure 1.6. Schematic presentation of the activation mechanism of $cPLA_2$. Phosphorylation of $Serine^{505}$ leads to catalytic activity of $cPLA_2$. Ca^{2+} ( ) , either released from endoplasmic reticulum (ER) or fluxed in during ischaemia due to Ca^{2+} homeostasis perturbations, binds to the N-terminal calcium-dependent-phospholipid-binding domain (CaLB) and promotes translocation to membranes (adapted from Hirabayashi and Shimizu, 2000).

amplitude and duration has been described, suggesting that the C2 domain regulates differential Ca^{2+} dependent membrane targeting (Evans *et al.*, 2001).

The C2 domain of $cPLA_2$ binds Ca^{2+} ions, spaced 4 Å, without inducing major conformational changes in the protein structure upon Ca^{2+} binding (Hirabayashi and Shimizu, 2000). The domain consists of an 8 stranded antiparallel ß - sandwich, which can coordinate between one and three Ca^{2+} atoms through a constellation of acidic residues on three Ca^{2+} binding loops. The function of the C2 domain is to present the catalytic unit to the substrate in a productive fashion (Dessen, 2000). Essential amino acid residues important for the catalytic mechanism of $cPLA_2$ are the Gly-Leu-Ser228-Gly-Ser segment, with the central Serine228 as active site (Kramer and Sharp, 1997). The $cPLA_2$ structure reveals a flexible lid or cap that must move to allow substrate access to the active site (Dessen *et al.*, 1999). The amphiphatic nature of the lid in $cPLA_2$ suggests that the enzyme can change from a soluble closed conformation to an open, more hydrophobic form stabilized by membrane binding (Dessen *et al.*, 1999). The highly basic, 180 residue cap, positions itself right between the active site, which is buried into the catalytic domain core, and the C2 domain, providing a surface patch for membrane contact (Dessen, 2000). In order for catalysis to occur, the substrate must travel from the bilayer into the depths of the active site, which can only happen if the hydrophobic rim of $cPLA_2$ is partially embedded in the target membrane (Dessen, 2000). Upon bilayer recognition, the catalytic unit is already strategically placed for substrate contact. A flexible inter-domain linker between the catalytic and the C2 domain as well as the lack of major protein interactions between them suggests the capacity of small rotations to accomplish optimal substrate targeting and optimal membrane interactions (Dessen, 2000 Dessen 99). The membrane binding itself is largely driven by hydrophobic interactions. It has been shown that the extent of membrane binding correlates with the amount of AA release (Hirabayashi and Shimizu, 2000).

3.4. $cPLA_2$ in the myocardium, IR injury and inflammation

PLA_2 have long been implicated in the pathogenesis of ischaemia and reperfusion injury and have attracted considerable interest as a pharmacological target in view of their involvement in lipid signalling and inflammatory conditions. In general, there

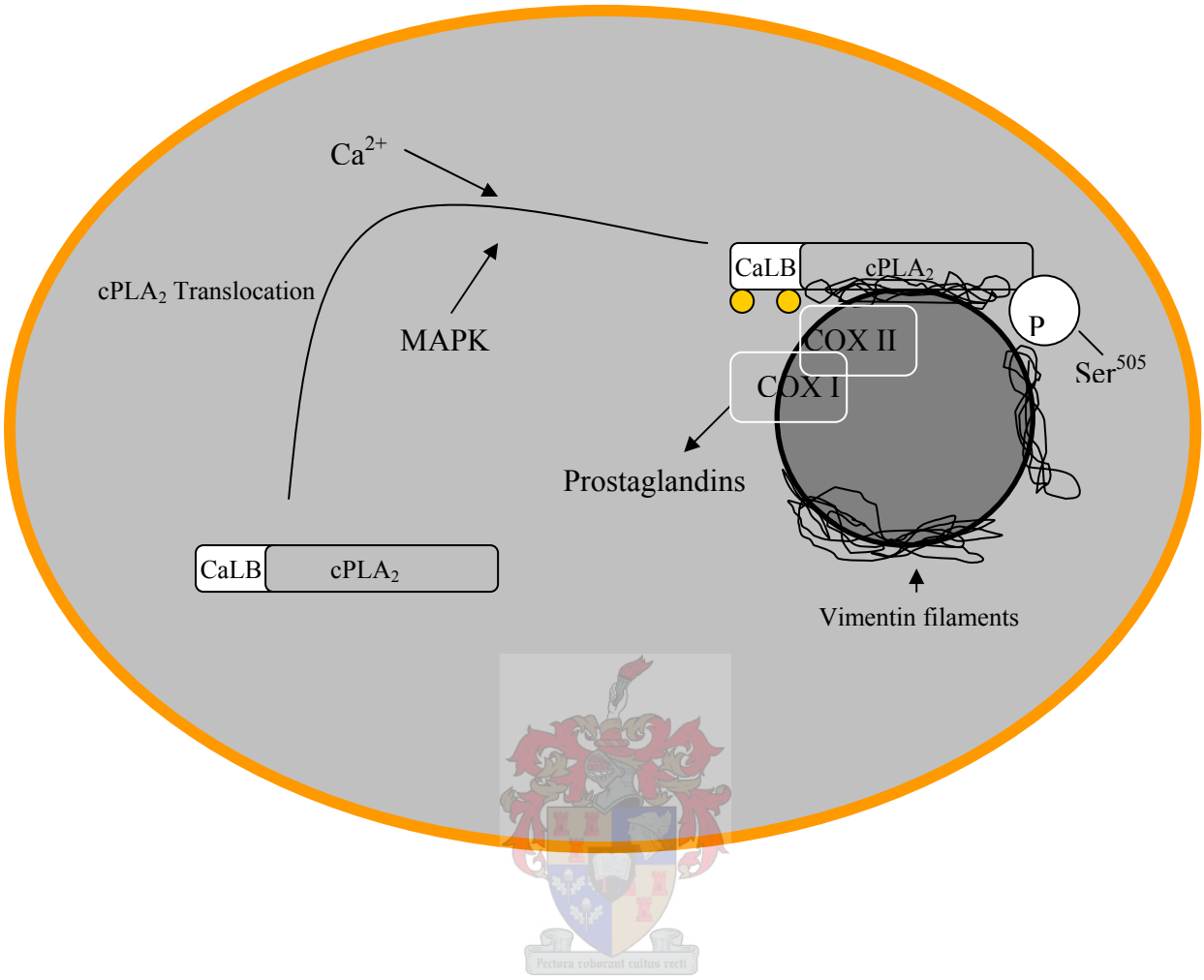


Figure 1.7. Translocation of $cPLA_2$ from cytosol to perinuclear membrane following Ca^{2+} mobilizing stimulus. Perinuclear enzyme profile cyclooxygenase I and II (COX I and COX II) assures proximity for inflammatory mediator synthesis. Vimentin filament acts as a functional adapter for $cPLA_2$ (adapted from Murakami *et al.*, 2000).

are several mechanisms for PLA_2 mediated injury, and due to the specific release of AA, $cPLA_2$ is of special interest regarding its inductive injury. The most remarkable evidence regarding the role of $cPLA_2$ in inflammatory mediator synthesis was shown in a $cPLA_2$ knock out model. $cPLA_2^{-/-}$ knock out mice subjected to 75 min focal cerebral ischaemia demonstrated a 34% reduction in brain infarct volume as well as less brain oedema and fewer neurological deficits than wild type mice (Sapirstein and Bonventre, 2000).

Global ischaemia and the high intracellular levels of free Ca^{2+} resulting from ischaemic membrane depolarisation and opening of Ca^{2+} channels are likely to trigger $cPLA_2$ activation (Kramer and Sharp, 1997). Cellular impairments during ischaemia, such as the acidification of the cytosol, disturbed Ca^{2+} homeostasis and alterations in physicochemical properties of the cellular membranes make the phospholipid constituents more vulnerable to $cPLA_2$ activity, and a vicious cycle starts. After only 10-20 min of ischaemia, increases in the $cPLA_2$ activity and protein levels have been shown (Saluja *et al.*, 1999). If preconditioning can be described as a lessening in calcium-mediated damage, then $cPLA_2$ should have its place within that pathway. Proinflammatory cytokines such as TNF-α produced during ischaemia induce activation and *de novo* synthesis of $cPLA_2$ and thus potentiate the mobilisation of $cPLA_2$. Therefore, they play a crucial role in the rapid and prolonged cellular responses occurring during inflammatory processes. Furthermore, the production and release of reactive oxygen species produced in ischaemia and reperfusion induce peroxidation of lipid membranes, which increases their susceptibility to the action of $cPLA_2$.

AA may affect a host of cellular processes directly and indirectly via downstream pathway recruitment. There is increasing evidence that alterations in the $cPLA_2$ activity are able to modulate myocardial AA levels. Firstly, the peroxidized metabolites of AA could exert inherent toxic effects. Secondly, the enzymes that metabolize AA produce reactive oxygen species (Burton *et al.*, 1990). Prostaglandins have been reported to exert both detrimental and beneficial actions on the ischaemic/reperfused heart, whilst leukotrienes are considered to induce negative actions on cardiac function, through vasoconstriction (Van der Vusse *et al.*, 1997). However, these conditions can be excluded in a cell culture setting, since no external

sources such as invading neutrophils are present. Interestingly, external AA administration to ischaemic neonatal cardiomyocytes proved to be cytoprotective (Engelbrecht *et al.*, 2005). Other reports have shown that cardiac phospholipid metabolism is compromised in the ischaemic heart. *In situ* experiments in canine heart showed that accumulation of AA is most prominent in the subendocardial layers of the ischaemic left ventricle, hence the region with the highest degree of underperfusion (Van der Vusse *et al.*, 1997). Restoration of flow to ischaemic tissue further increases cardiac AA levels. Furthermore, the post-ischaemic accumulation of AA correlates positively with lactate dehydrogenase, a marker for irreversible loss of cellular integrity, suggesting a relation between the degree of ischaemic damage and the extent of AA accumulation (Van der Vusse *et al.*, 1997). AA administration affected the amplitude of the Ca^{2+} transient in cardiac myocytes and reduced the contraction rate (Van der Vusse *et al.*, 1997). Furthermore, myocardial ischaemia results in accelerated phospholipid catabolism, which leads to the accumulation of amphiphilic metabolites in critical subcellular loci, which precipitate electrophysiologic dysfunction and myocyte cell death (Hazen *et al.*, 1991).

On the other hand, it has been suggested that the overwhelming majority of measurable phospholipase A_2 activity in the heart is the calcium-independent $iPLA_2$ isoform (McHowat and Creer, 2004). A 10 fold induction of $iPLA_2$ during brief myocardial ischaemia has been reported (Hazen *et al.*, 1991). The complexity of the signalling cascade and the identification of numerous mechanisms for $cPLA_2$ activation are further complicated by the overlapping expression of multiple PLA_2 enzymes with interdependent actions within the cell. For example it has been suggested that $cPLA_2$ might be important in modulating the activity of $iPLA_2$ (McHowat and Creer, 2004).

The $cPLA_2^{-/-}$ mouse has demonstrated the importance of $cPLA_2$ in various physiological processes. Despite profound defects in the fertility of female $cPLA_2^{-/-}$ mice, macrophages derived from these mice failed to make leukotrienes and prostaglandins. In septic patients, plasma PLA_2 activity is elevated and is correlated with the severity of organ dysfunction and mortality. When sepsis is induced and measured in the rat heart, a 60% $cPLA_2$ activity increase was observed during late sepsis (Tong *et al.*, 1998). Recently, a safeguarding role of $cPLA_2$ has been suggested,

due to its pathway coupling to ß2-adrenergic receptors in the human heart. Upon ß2-adrenergic receptor stimulation, $cPLA_2$ translocates to caveolae functional platforms where it facilitates phosphorylation of endothelial nitric oxide synthase (Ait-Mamar *et al.*, 2005).

4. Mitogen activated protein kinases (MAPK) and ischaemic preconditioning

4.1. Introduction

Several protein kinase pathways are activated during cell injury caused by myocardial ischaemia and ischaemia/reperfusion. They capacitate in the onset of cell injury and the reduction of this injury by ischaemic and pharmacologic preconditioning. One of the primary pathways involved in this context is the MAPK pathway. MAPKs are a conserved family of enzymes which transduce extracellular signals into intracellular responses. The MAPK pathway is activated by ligand binding to receptor-associated tyrosine kinases (RTKs) and requires the activation of guanidine triphosphate- (GTP) binding proteins (Lazou *et al.*, 1998). Receptor binding causes RTK monomer dimerisation and phosphorylation of tyrosine residues on the cytosolic domain (Chakraborti and Chakraborti, 1998). This activation stimulates the cytoplasmic MAPKs sequentially (Chakraborti and Chakraborti, 1998). Each pathway follows the same conserved module, namely: a MAPK kinase kinase (MEKK) is activated and phosphorylates a MAPK kinase (MEK or MKK) via serine/threonine phosphorylation. MKK is a dual specificity kinase and phosphorylates MAPK at a threonine and tyrosine residue. All of the MAPKs are activated by dual phosphorylation of a serine and a threonine residue by a MAPK kinase (Michel *et al.*, 2001). The three major MAPKs in the myocardium are the 42 and 44 kDa extracellular signal-regulated kinases (ERK1/2), as well as the two stress-activated 46 and 54 kDa c-Jun N- terminal kinases (JNK 1/2) and the 38 kDa p38 kinases (Cohen *et al.*, 2000) . Not only are they role players in preconditioning (Maulik *et al.*, 1996; Clerk *et al.*, 1998)**,** but p38 and ERK also have the capacity to phosphorylate $cPLA_2$ and thus contribute to its optimal activity (Gijon *et al.*, 1999). Therefore, these two MAPK deserve special attention within the scope of this study.

4.2. MAPK activation and ischaemic preconditioning

4.2.1. ERK activation and ischaemic preconditioning

It has been shown that ERK is activated in ischaemia, however, a fundamental role for ERK activation in cardioprotection is controversial. It is known that ERK mediates cell growth and survival signals (Burgering and Bos, 1995). The role of ERK as a potential mediator of IPC has been controversial although the majority of studies suggest a role for ERK in IPC (Omura *et al.*, 1999; Liu *et al.*, 2003). PKC is the upstream activator of ERK as well as ROS. In response to a preconditioning stimulus, ERK translocates to the nucleus, where it has been demonstrated to activate NFkB and AP-1 (Hobbie *et al.*, 1997; Hausenloy and Yellon, 2006). In IPC two phases of ERK activation have been described, the first one immediately post-IPC stimulus and the second one at reperfusion. When inhibiting the first activation phase, the second one was abolished, providing evidence that phase one activation might serve as an execution requirement for ERK phosphorylation at reperfusion. Activation of ERK-1/2 at reperfusion, following a prolonged ischaemic event, protects the heart against ischaemia/reperfusion injury (Hausenloy *et al.*, 2004). Hausenloy *et al*. (2004) demonstrated a 4 fold increase in ERK activation at 15 min into reperfusion, which was preceeded by a 35 min ischaemic period. It has been shown that inhibition of ERK enhances ischaemia/reperfusion induced cell death, whilst a sustained activation contributes to adaptive cytoprotection in cultured neonatal cardiomyocytes (Yue *et al.*, 2000). Liu and co-workers (2003) have demonstrated that ERK is strongly activated in neonatal cardiomyocytes at 10 min and 30 min after hypoxic preconditioning with a downregulation of phosphorylation at 60 min post hypoxic preconditioning (Liu *et al.*, 2003). In this model the protective effects of hypoxic preconditioning were completely abolished by PD98059, a selective inhibitor of MEK-1/2, an upstream activator of ERK.

4.2.2. p38 activation and ischaemic preconditioning

Most focus has been on the p38 MAPK cascade with most controversy and confusion regarding its scope of action in the context of IPC (Steenbergen, 2002). Two of the at least 6 known p38 isoforms, namely p38α and p38β are expressed in cardiac muscle.

p38 is activated by sustained ischaemia followed by reperfusion as well as by short episodes of ischaemia. However, p38´s contribution to benefit the ischaemic heart is controversial. Some studies have shown that preconditioning activates p38 MAPK during ischaemia and when inhibiting it, protection is lost, suggesting a clear protective role of p38 in the preconditioning context (Yellon and Downey, 2003). Within the course of ischaemia, however, the level of p38 is reduced towards pre-ischaemic values, followed by another phosphorylation period at reperfusion. It is of importance that in ischaemia in non preconditioned hearts no p38 activation *per se* is observed. Furthermore, in many studies it was shown that inhibition of p38 leads to a higher degree of resistance against ischaemic injury in non-preconditioned tissue. To add to the confusion, p38 inhibition blocked preconditioning induced protection (Yellon and Downey, 2003).

Yue and co-workers (2002) have shown protection in isolated rat hearts with the p38 activator menadione. They suggested a role for ROS which could activate p38 in a similar manner. p38, in turn through phosphorylation of the HSP27, would stabilize stress fibres and thereby conferring resistance to ischaemic associated osmotic swelling, associated with ischaemia in the heart (Yue *et al.*, 2002). It was also shown that myocardial ischaemia and reperfusion activate p38 MAPK *in vivo* (Feuerstein and Young, 2000). It was demonstrated that ischaemia alone increases p38 moderately by 3,5 fold over the baseline while reperfusion further increases p38 MAPK by 6,3 fold in the rabbit heart, perfused via the Langendorff system (Feuerstein and Young, 2000). Interestingly, activation of p38 is a rapid event occurring within minutes, and precedes cellular and organ lesions. Importantly, administration of the p38 MAPK inhibitor SB203580 before the ischaemic event improved functional parameters: it accelerated the recovery of coronary flow, contractility and left ventricular pressure (Feuerstein and Young, 2000).

The described results in the literature make it clear that different models such as simulated ischaemia, ischaemia/reperfusion and hypoxia have a differential potency and outcomes. The investigation of hypoxia and hypoxia/reoxygenation of rat neonatal ventricular myocytes by Seko and co-workers (1997) revealed that both conditions caused rapid p38 activation (Seko *et al.*, 1997). Here, the p38 phosphorylation reached maximum levels after 2 to 5 min hypoxia and again after 5 to

10 min reoxygenation (Seko *et al.*, 1997). Other studies, on the other hand, have demonstrated that IPC reduces p38 activity during ischaemia, and pharmacological inhibition of p38 activity during ischaemia/reperfusion is cardioprotective (Marais *et al.*, 2001). This implies a detrimental role of p38 MAPK in ischaemia/reperfusion.

Taking these findings together, IPC can activate or reduce p38 MAPK during the prolonged ischaemic event. A generally accepted view is that IPC transiently activates p38 MAPK during the preconditioning phase and reduces its activation during the prolonged ischaemic event. Four isoforms of p38 have been described: p38α, p38β, p38γ and p38δ. The different p38 isoforms, their expression pattern and different experimental models and protocols, as well as usage of different species have been suggested to be the reason for the conflicting findings (Yellon and Downey, 2003). It has been implied that different p38 isoforms mediate different adaptive responses. Both p38α MAPK and p38β MAPK mRNA have been detected in the heart. Evidence suggests that p38α MAPK activation may be coupled to apoptosis whereas p38β MAPK activation may be coupled to protective cellular responses (Wang *et al.*, 1998; Saurin *et al.*, 2000). This is in agreement with the observation that overexpression of p38α results in apoptosis in cardiomyocytes (Wang *et al.*, 1998). Others explain the differential outcome with differential p38 activation dynamics and suggest that the duration of p38 activation determines its cellular function. Mackay and co-workers (2000) showed that extended p38 activation during ischaemia leads to cell death in neonatal rat cardiomyocytes (Mackay and Mochly-Rosen, 2000). They demonstrated a higher susceptibility to cell death from ischaemia in a dose and time dependent manner by modulating p38 activity with a tyrosine phosphatase inhibitor (Mackay and Mochly-Rosen, 2000).

4.2.3 JNK activation and ischaemic preconditioning

The role of JNK in IPC is also controversial, since both protective and detrimental activities have been described. The majority of reports suggest that JNK is activated only during reperfusion after ischaemia but not affected by ischaemia alone (Sugden and Clerk, 1998). Sato and co-workers (2000) describe an increase of JNK in isolated rat hearts exposed to 30 min ischaemia followed by 2 h of reperfusion (Sato *et al.*, 2000). In anesthetized rabbits submitted to a preconditioning protocol JNK's

phosphorylation was observed after 20 min of sustained ischaemia (Iliodromitis *et al.*, 2002). In ischaemically injured porcine ileum mucosa as a model for intestinal ischaemia, it was shown that ERK1/2 and p38 positively regulate recovery from ischaemia, whilst JNK mediates negatively recovery from ischaemia (Shifflett *et al.*, 2004).

In summary, although literature supports a potential role for ERK, p38 MAPK and JNK in preconditioning (Clerk *et al.*, 1998), contrary reports are available. These conflicting results may be due to the use of different experimental models. Due to the discussed role of the different MAPKs in preconditioning and their differential capacity to phosphorylate $cPLA_2$, we did not look into more detail at JNK activation.

5. Myocyte cell death

5.1. Introduction

Two principal avenues leading to myocyte death have been described, namely necrosis and apoptosis. Necrosis is characterized by cell swelling and blebbing and disruption of the sarcolemmal and mitochondrial membrane (Clerk *et al.*, 2003). Furthermore, active transmembrane ion transport systems are slowed down, leading to increased cell membrane permeability with alterations in ion distribution for K^+, Na^+ and Ca^{2+}. This loss of ion concentration gradients leads to loss of control of cell volume regulation, causing swelling of cell organelles which will lead to their disruption and subsequent lysis (van Wijk and Hageman, 2005). Also, activated proteases and elevated Ca^{2+} levels may alter mitochondrial respiratory function causing increased production of reactive oxygen species (van Wijk and Hageman, 2005). This unregulated process produces cellular debris and subsequently leads to the induction of inflammation.

Apoptosis, on the other hand, is an extremely well regulated and controlled form of cell death – therefore, termed programmed cell death - which results in a systematic cellular dismantling without inducing inflammation. Cells undergoing apoptosis have characteristic morphological features such as nuclear condensation and fragmentation

as well as the formation of apoptotic bodies (Clerk *et al.*, 2003). A key phenomenon of apoptotic cell death is the activation of a unique class of aspartate specific proteases known as caspases. Caspases can be grouped in an upstream (effector caspases-1, -2, -4, -5, -8, -9, -10) and a downstream (executioner caspases-3, -6, -9) subgroup. One of the substrates for caspase-3 is the repair enzyme PARP, yielding a 85 kDa inactive fragment which is a useful marker for activation of caspases during apoptosis (Piot *et al.*, 1999). The function of caspase-3 mediated cleavage of PARP is to protect the pool of energetic substrates against the use of these substrates by PARP (Yang *et al.*, 2000). Cleavage of PARP by caspase-3 during apoptosis facilitates nuclear disassembly and may help to ensure the completion of apoptosis. Therefore, when PARP is cleaved by caspases, the ATP "wasting" repair attempt is paused and apoptosis can be executed (Szabo, 2005). Importantly, a systemic inflammatory response is prevented.

How much decision making the cell capacitates in choosing a specific death avenue depends on the intracellular environment, such as the metabolic status and energy content, the pH and [Ca^{2+}] and the redox potential, all dictating the final outcome of cellular fate (Clerk *et al.*, 2003).

5.2. PARP activation-shift towards necrosis

In contrast to PARP cleavage, PARP activation induces necrotic cell death. The obligatory trigger for its activation is nicks and breaks in the DNA strand. The generation of free radicals and oxidants in cardiac myocytes during ischaemia/reperfusion leads to such DNA strand breakage. This initiates an energy consuming and inefficient metabolic cycle with transfer of the ADP-ribosyl moiety of NAD^+ to protein acceptors. Resynthesis of NAD^+ requires ATP and poses a heavy demand on the cellular energy capacities. Failure to overcome this crisis leads to cell death, apoptotic or necrotic, depending on ATP availability. In this way, ATP is rapidly diminishing and its usage for other processes, including apoptosis is prevented. Therefore, the energy depletion caused by PARP activation induces rapid necrotic rather than delayed apoptotic cell death, in other words, PARP activation shifts cellular death towards necrosis, away from apoptosis. In this way PARP may

prevent several damaged cells from attempting to repair themselves and surviving with a high mutation frequency (Martin *et al.*, 2005).

PARP activation due to ROS, such as superoxide anions, hydroxyl radicals and hydrogen peroxide contributes to myocardial and vascular injury. In H9c2 cardiac myoblasts it has been shown that oxidant injury via hydrogen peroxide application causes PARP activation with subsequent reduction in mitochondrial respiration. This suppression was ameliorated when pharmacologically inhibiting PARP. Also, hypoxia and reoxygenation, without other stress components used in simulated ischaemia, resulted in PARP activation. Furthermore, PARP activation in neonatal rat cardiac myocytes has been observed in nitric oxide induced cell necrosis, which was also ameliorated by pharmacological PARP inhibition (Szabo, 2005).

The activation of PARP can lead to massive NAD^+ usage that correlates with changes in the cellular NAD^+ amount (Szabo and Dawson, 1998). Although necrosis induction is fast, it induces a systemic inflammatory response. This scenario can only be avoided through cleavage and inactivation of PARP.

5.3. Caspase- and PARP cleavage in context of I/R injury

Both ischaemia and reperfusion increase the production of ROS, the first through mitochondrial respiratory mechanisms, the second through degradation of energetic substrates (van Wijk and Hageman, 2005). ROS react directly with biological molecules, such as lipid membranes, proteins and DNA and create a disturbance in ROS homeostasis and a further generation of ROS (van Wijk and Hageman, 2005). ROS produced during ischaemia and reperfusion induce DNA strand breaks which leads to over-activation of PARP.

It was also shown in ischaemia/reperfusion experiments, that the reoxygenation induced suppression of the myocardial contractility is dependent on the functional integrity of PARP. The activation of PARP might be contributing to the development of reperfusion injury in the heart (Szabo and Dawson, 1998) since it creates a non-beneficial energetic environment for the cell. Multiple lines of evidence implicate PARP in the pathogenesis of heart failure. In fact, one of the best characterized

pathways is PARP activation in relation to myocardial energy metabolism. Within the muscle contraction context, myocardial contraction is especially tightly regulated. Disruption of cellular or mitochondrial energetics leads to elevated intracellular Na^+ and Ca^{2+} levels and progressive acidosis (Szabo, 2005).

In the ischaemia/reperfusion setting a rather prolonged time course of PARP activation has been observed, present at 1 h after start of reperfusion and continuously present as late as 24 h after reperfusion. Cell death in this context can either be of necrotic or apoptotic nature or a combination of both. Especially the time of apoptosis initiation is still to a large extent unknown. In experiments exposing cells to 6 h ischaemia without reperfusion showed no DNA fragmentation (Freude *et al.*, 2000). Therefore, it has been hypothesised that the "point of no return" could be situated farther downstream in the apoptotic cascade. Forty five min of ischaemia resulted in reversible injury and 90 min in irreversible ischaemic injury. In irreversible injury, caspase-3 activation and PARP cleavage were present after ischaemia whilst in reversible injury, neither caspase-3 activation, nor PARP cleavage was present (Freude *et al.*, 2000). It can be deducted that activation of caspase-3 and cleavage of PARP are early events in ischaemia; and that DNA fragmentation occurs only in late reperfusion, representing the end-stage within the apoptotic cascade (Freude *et al.*, 2000). Therefore, inhibition of apoptosis should be undertaken early in ischaemia.

In neonatal cardiomyocytes, simulated ischaemia induced caspase-3 activation and PARP cleavage, preceded by any morphological signs of apoptosis, confirming that the apoptotic cascade is activated early during ischaemia, after 5 min, but is executed only after reperfusion (Engelbrecht *et al.*, 2004).

When preconditioning rat hearts with five 5 min cycles followed by 30 min of coronary occlusion and 180 min reperfusion, cleavage of caspase-3 and inactivation of PARP were prevented (Piot *et al.*, 1999). It was also found that apoptosis was reduced by preconditioning rabbit cardiomyocytes. These data suggest that IPC reduces irreversible ischaemic injury partly by decreasing apoptosis after prolonged ischaemia and reperfusion.

The molecular mechanisms by which caspases carry out the execution of apoptosis are complex and require many enzymes and cofactors. However, the caspase dependent apoptotic pathway is not the only one indicated in I/R injured tissues. In the presence of the overall caspase inhibitor Z-DEVD-FMK, which is able to inhibit

caspase-1, -3 and -4, nuclear fragmentation was also present and although reduced, significant tissue damage was present (van Wijk and Hageman, 2005). Therefore, caspase-independent pathways have been suggested. On the other hand Z-DEVD-FMK attenuated ischaemia/reperfusion injury in rat hearts (van Wijk and Hageman, 2005).

5.4. PARP in the context of inflammation

Of special interest is the fact that PARP can exert profound effects on inflammatory mediators and downstream processes. Recent studies have implicated PARP in local as well as systemic inflammatory conditions. PARP inhibition has been shown to suppress TNF-α production in the late stage of myocardial reperfusion injury. Furthermore, inhibition of PARP suppresses the development of rheumatoid arthritis in rodent models and improves the survival rate of mice injected with a high dose of endotoxin.

These findings are surprisingly similar to the earlier discussed context of TNF-α and inflammation in the myocardium and in rheumatoid arthritis. One can expect that PARP dependent regulation of NF-κB activation has a broad effect on the expression of proinflammatory genes. *In vitro* and *in vivo* experiments indicate that inhibition of PARP activation exerts effects on the expression, activation and nuclear translocation of key proinflammatory genes and proteins. (Yang *et al.*, 2000) The absence or inhibition of PARP suppresses the activation of MAPKs and NFκB. Consequently, PARP inhibition interferes with the expression of proinflammatory genes such as the inducible nitric oxide synthase (iNOS). Furthermore, inhibition of PARP is associated with a reduction in neutrophil infiltration in the site of injury in myocardial reperfusion. This is of importance since these cells mediate the TNF-α response from the systemic side.

5.5. Clinical application

Evidence suggests that inhibition of PARP is beneficial, especially in the early phase of myocardial reperfusion injury. Genetic disruption of PARP provides marked protection against delayed myocardial ischaemia and reperfusion injury (Yang *et al.*,

2000). Due to the central role of PARP in the decision making of cell death, a primary objective would be to shift cell death from necrosis towards apoptosis, for two reasons. Firstly, apoptosis buys out lethal time, which "in the mean time" would have been "used" for a fast necrotic cell death. This increases the chance to rescue the tissue, if the "point of no return" in the death cascade has not yet been reached. It implies however, that reperfusion injury needs to be understood in the context of various stages in the apoptotic cascade. Secondly, it prevents a systemic response involving the whole organism, which via the inflammatory cascade would provide positive feedback into the tissue of injury, as seen in cytokines and heart failure. PARP-1 has currently reached the stage of phase I/II clinical testing as cardioprotector and sensitizer for chemotherapy (Graziani *et al.*, 2005).

6. Crosstalk

6.1. Introduction

In ischaemia/reperfusion injury, increased cellular Ca^{2+} and the production of reactive oxygen species (ROS) may cause cell death by intrinsic apoptotic pathways or by necrosis. Both the activity of $cPLA_2$ and the actions of TNF-α are closely interwoven within this context. We have dealt with their individual roles in ischaemic injury, but one has to consider a crosstalk between them, since this mirrors the more cell physiological approach. Can a molecular link be established between TNF-α and $cPLA_2$ signalling, directing the decision making of cellular death via PARP and caspase-3? On several points within the stress response pathways, the key signalling components, viz TNF-α, $cPLA_2$, MAPK, caspase-3 and PARP interlink or overlap, especially within the context of inflammation and apoptotic cell death. Therefore, in this chapter I would like to shed light onto possible interdependencies of these role players, whether they jointly modulate the cytoprotective effect as described in TNF-α preconditioning.

6.2. TNF-α receptor binding directs apoptosis and inflammation

6.2.1 Inflammation via $cPLA_2$

The most potent stimuli that elicit apoptosis in cardiomyocytes are amongst others oxygen radicals, cytokines, stress conditions such as ischaemia, (especially when followed by reperfusion) as well as physical or chemical stress and sphingolipid metabolites (Feuerstein and Young, 2000). Cardiac myocyte apoptosis varies between 0.1-30% depending on the disease specimen, methodology and model employed as well as area of sampling (Feuerstein and Young, 2000). In general, apoptosis is not found with inflammation, as these two conditions, the pro-apoptotic and proinflammatory effects of TNF-α can be seen as mutually exclusive. It has been suggested that caspases are the molecular tool used by TNF-α to control the proinflammatory response. Most cellular TNF-α responses, including $cPLA_2$ activation, are mediated by TNFR1 and CD95. Both of these receptors also mediate apoptosis, whilst the important proinflammatory mediator AA is generated via $cPLA_2$. The adapter proteins on the cytosolic side of the receptor provide a modularity that allows flexible regulation of signalling events which needs to direct the two conflicting pathways: apoptosis versus inflammation (Kronke and Adam-Klages, 2002). If the ATP dependency in an ischaemically challenged cell plays a role in shifting apoptotic death towards necrotic death, then this exact receptor modularity should be a crucial decision maker between life and death for the cardiomyocyte. It has been suggested that TNF-α induces $cPLA_2$ phosphorylation and activation, which is triggered through the death domain and involves adapter proteins that result in the activation of Raf-1 and ERK (Kronke and Adam-Klages, 2002) (Figure 1.8.). This mode of action would mediate the inflammatory response. The involvement of MAP kinases in the control of $cPLA_2$ and AA release has been described by Gudmundsdottir *et al* (2001). They have found that agonist stimulated endothelial cells treated with MAPK inhibitors, PD98059 (ERK inhibitor) and SB203580 (p38 inhibitor), showed decreased arachidonic acid release and $cPLA_2$ activity. Further evidence substantiating the role of ERK activation through TNF-α was provided by Lüschen and co-workers (2000), who have demonstrated that TNF-α dependent activation of ERK and $cPLA_2$ requires the intact death domain of the receptor TNF-R55 (Luschen *et al.*, 2000). The same team has also shown that in murine fibroblasts

$cPLA_2$ is phosphorylated in response to TNF-α by ERK only, not by p38 MAPK, suggesting a molecular link from the receptor via the death domain to ERK and $cPLA_2$ (Luschen *et al.*, 2000). It has been shown that MAPK-activating death domain protein (MADD) and receptor-interacting protein 2 (RIP2) can interact with the death domain to mediate ERK activation (Luschen *et al.*, 2000). Furthermore, it was shown in a murine fibroblast cell line that inhibition of p38 enhanced TNF-α induced cell death and led to the activation of caspase-3. Overexpression of p38 did not protect the cells from TNF-α induced cytotoxicity (Luschen *et al.*, 2004).

6.2.2. Apoptosis via $cPLA_2$ cleavage

Interestingly, $cPLA_2$ belongs to the family of enzymes which can be cleaved by certain caspases, namely by caspase-3, -7 and -8. This renders a cleavage product of 70 and 32 kDa after cleavage of the caspase-3 motif at amino acids 519-522. This control mechanism could be responsible for TNF-α mediated cytoprotection, escaping the detrimental inflammatory response leading to necrotic cell death, but rather shifting the stress response towards apoptosis. The multiple $cPLA_2$ cleavage sites for various caspases would ensure its cleavage, thus leading to apoptosis. It has been shown that cleavage of $cPLA_2$ resulted indeed in the inactivation of its enzymatic activity (Kronke and Adam-Klages, 2002). In TNF-α stimulated human embryonic kidney (HEK) cells an increase in $cPLA_2$ activity was seen after caspase-3 inhibition, suggesting a role for caspases to downregulate or terminate $cPLA_2$ activation. Interestingly, caspase-3 has been shown to be essential for vimentin proteolysis (Slee *et al.*, 2001), a structural protein associated with $cPLA_2$ translocation (Murakami *et al.*, 2000).

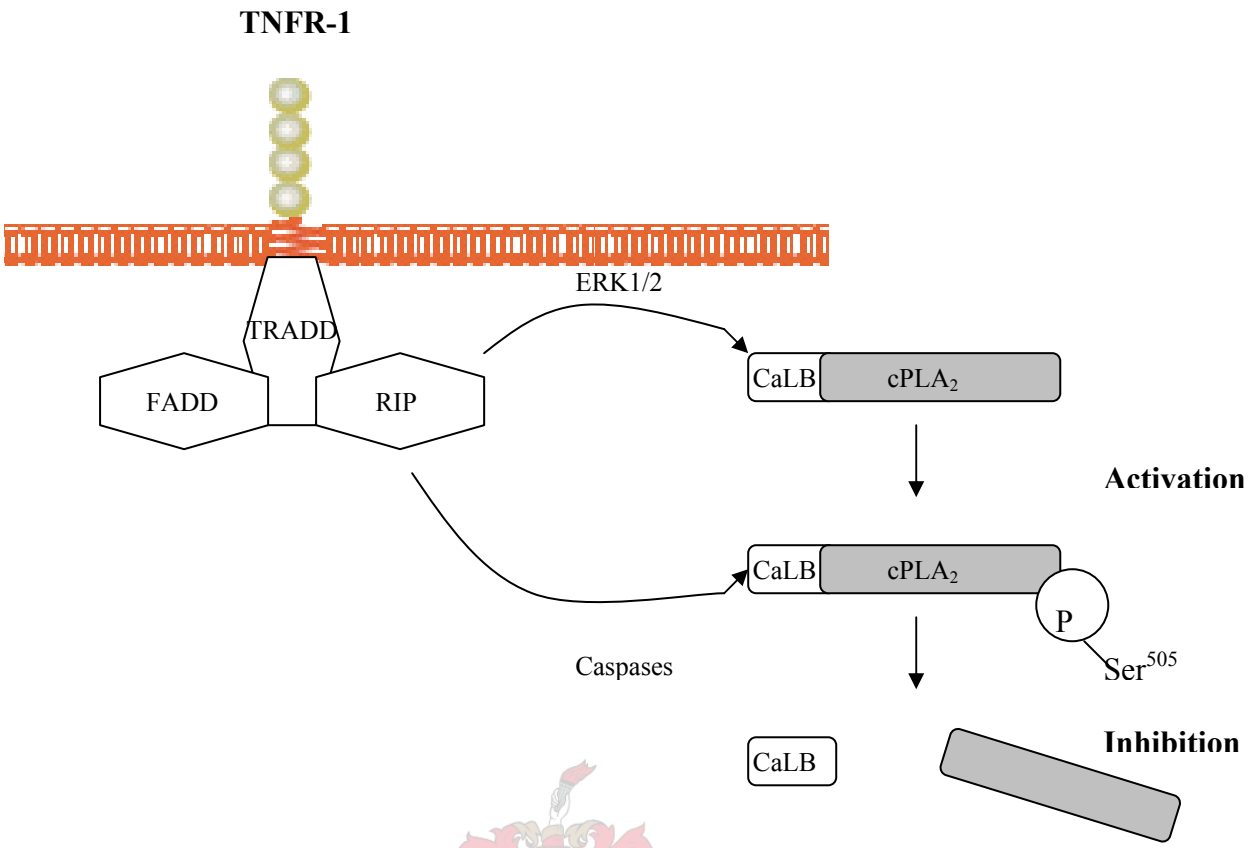


Figure 1.8. TNF-α mediated activation and inactivation of $cPLA_2$ (modified from (Kronke and Adam-Klages, 2002).

6.3. Modulation of contractility and gene expression via TNF-α and $cPLA_2$

Another point of signalling overlap between $cPLA_2$ and TNF-α has been shown by Amadou and co-workers (2002). Investigating the role of AA as mediator of TNF-α on Ca^{2+} transients and contractility in rat cardiomyocytes, the key role of $cPLA_2$ and the AA pathway in mediating contractile effects of TNF-α is apparent, with a TNF-α dose dependent outcome (Amadou *et al.*, 2002). 10 ng/ml TNF-α produced a 40% increase in the amplitude of Ca^{2+} transient and myocyte contraction, whilst a higher dose (50 ng/ml) TNF-α evoked a biphasic effect, an initial positive effect followed by a sustained negative effect. Both the positive and negative effects could be reproduced by AA and blocked by $AACOCF_3$ (10 μM), pre-incubated for 15 min. Another study substantiating the crosstalk between $cPLA_2$ and TNF-α, even on the gene expression level, was shown by Thommessen and co-workers (1998). They demonstrated that TNF-α induced activation of NF-κB was inhibited by a selective $cPLA_2$ inhibitor $AACOCF_3$ (Thommesen *et al.*, 1998). A human skin keratinocyte cell line which was pre-treated for 1h with $AACOCF_3$, before stimulating with 10 ng/ml TNF-α, showed a 70% reduction in NFκB activation (Thommesen *et al.*, 1998).

6.4. $cPLA_2$, AA and apoptosis

Programmed cell death is associated with changes in glycerophosholipid metabolism. In some cells undergoing apoptosis, AA release parallels the reduction in cell viability and DNA fragmentation (Taketo and Sonoshita, 2002). AA release has also been associated with TNF-α induced apoptosis. It has been shown that $cPLA_2$ is critical in the cytotoxic actions of TNF-α via metabolites of AA. ROS are generated by the peroxidation of arachidonic acid metabolites. This fast available source for oxygen radicals is thought to be involved in TNF-α induced cytotoxicity. TNF-α rapidly stimulates PLA_2 within 5-10 minutes post-receptor binding and subsequently increases AA release (Prabhu, 2004). AA might be responsible for the dual contractile effect of TNF-α, since PLA_2 inhibition abrogates both the positive and negative inotropic responses induced by TNF-α. AA initially induced a direct cardiostimulatory effect which was followed by sphingomyelinase activation and downstream negative inotropy. *In vitro* and isolated cell studies indicate that cytokine

mediated PLA_2 activation precedes sphingomyelin hydrolysis and that AA activates sphingomyelinase.

6.5. Aims and Hypothesis

Taking the findings of this literature survey into synthesis, a crosstalk of the mentioned role players is apparent. The aim of the present study is to investigate this exact crosstalk of $cPLA_2$ in the context of TNF-α as a preconditioning mimetic. We hypothesize a role for $cPLA_2$ in TNF-α mediated cytoprotection, possibly on the level of $cPLA_2$ activation and phosphorylation, or through a specific $cPLA_2$ translocation.

The role of $cPLA_2$ will be described in a threefold manner:

- its activation via phosphorylation,
- its translocation to sub-cellular structures
- its turnover through caspase mediated cleavage

The interplay of $cPLA_2$ with the MAPKs p38 and ERK as possible contributors to $cPLA_2$ phosphorylation and activation will also be investigated. Sub-cellular $cPLA_2$ translocation will be monitored and co-localized with the nuclear envelope and the endoplasmic reticulum. Furthermore, $cPLA_2$ in the context of ischaemically induced cell death will be evaluated, monitoring cell viability as well as caspase-3 and PARP activation. At the same time this will allow us to investigate $cPLA_2$ turnover regulation via caspase-3 mediated $cPLA_2$ cleavage. This will shed light on the survival mechanisms employed by the ischaemically challenged cell in a setting of TNF-α mediated cytoprotection.

Chapter 2: Materials and Methods

2.1. Materials and Reagents

Highly purified recombinant murine TNF-α was purchased from CytoLab (Israel), the $cPLA_2$ inhibitor arachidonyl trifluoromethyl ketone ($AACOCF_3$) from Calbiochem (USA) and the caspase inhibitor Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK) from SIGMA-ALDRICH (South Africa). The following antibodies as well as the caspase-3 transfection- and silencing kit were obtained from Cell Signaling Technology (MA, USA): $cPLA_2$ antibody and phospho-$cPLA_2$ antibody (Ser 505), p38 MAPK antibody and phospho-p38 MAPK (Thr180/Tyr182) antibody, p44/42 MAPK antibody and phospho-p44/42 MAPK (Thr202/Tyr204) antibody, cleaved caspase-3 antibody (Asp 175) and PARP antibody. Antibodies to nucleoporin p62 and calnexin were purchased from Santa Cruz Biotechnology (California, USA). Tissue culture tubes, 50 ml and 15 ml were used from Greiner Bio-One (Germany). Sterile serological pipettes (25ml and 10ml) were obtained from LP Italiana SPA and Sterilin Ltd. Pipette tips were purchased from Greiner Bio-One. Culture medium, Dulbecco's modified Eagle's medium (DMEM) was obtained from Highveld (South Africa), trypsin and phosphate buffered saline (PBS) from SIGMA-ALDRICH (South Africa) and tissue culture flasks and -dishes, cell scraper and microcentrifuge tubes were purchased from Greiner Bio-One (Germany). Eight chamber slides were used from Nunc Lab-Tek (USA). Syringe milipore filter, trypan blue reagent, albumine bovine serum (BSA) and Bradford reagent were obtained from SIGMA-ALDRICH (South Africa). For the gel electrophoresis a Mini-Protean BIO RAD system was used. Ammonium persulfate was purchased from SIGMA-ALDRICH (South Africa), Acrylamide/bis-Acrylamide from Promega (South Africa) and N,N,N,N'-Tetramethylethylenediamine from MERCK (Germany).

2.2 Cell Culture

C2C12 mouse myoblasts (European Collection of Cell Cultures - ECACC), seeded at 12 000/cm^2, were cultured in Dulbecco's modified Eagle's medium supplemented

with 10 % fetal calf serum, 4% glutamine and 1% penicillin/streptomycin in a humidified atmosphere, 37°C, in the presence of 5% CO_2. In detail, cells were washed with 0.01 M sterile PBS, typsinized (0.25% Trypsin – EDTA), centrifuged for 3 min at 6000 x g and seeded as follows: $3x10^5$ myoblasts per 25 cm² tissue culture flask, $1x10^5$ myoblasts per culture dish in six-well plate and $2x10^4$ myoblasts per 8 chamber slide. Myoblast differentiation was induced when cells reached approximately 70% confluency, by replacing the growth medium with a low serum differentiation medium (DMEM supplemented with 1 % filter sterilized horse serum). Differentiation medium was changed every 48 hours.

2.3. Experimental protocol

On day 8, 9 and 10 of differentiation, terminally differentiated myotubes were randomly divided into 6 groups: 1. control (Con), 2. simulated ischaemia (SI), 3. ischaemic preconditioning (IPC), 4. TNF-α preconditioning (TNF-α PC), 5. TNF-α + inhibitor ($AACOCF_3$ or Z-DEVD-FMK) and 6. inhibitor only ($AACOCF_3$ or Z-DEVD-FMK).

Control cells were kept under normoxic conditions and 5% CO_2. The period of simulated ischaemia (1% O_2, 5% CO_2) lasted for 8 hrs. IPC cells were washed with sterile PBS and prior to the simulated ischaemic event pre-incubated for 30 min with filter sterilized modified Esumi buffer (Esumi *et al.*, 1991), pH 6.2, containing (in mM): 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 Hepes, under hypoxic conditions, 1% O_2 and 5% CO_2, followed by a 60 min washout time under normoxic conditions and differentiation medium, before submitted to the simulated ischaemic event. TNF-α PC cells were pre-incubated with 0.5ng/ml TNF-α in differentiation medium under normoxic conditions, followed by a 60 min washout period before the simulated ischaemic event. The same procedure was followed for group 5 and 6, (0.5ng/ml TNF-α + 10μM $AACOCF_3$/10μM Z-DEVD-FMK or the inhibitors only) respectively. Following the washout phase, cells were washed twice with PBS and submitted to the simulated ischaemic event, using modified Esumi buffer + 20 mM 2-Deoxy-D-Glucose (2-DG) pH 6.4 (Figure 2.3.). 2-DG inhibits glycolysis irreversibly and limits cellular metabolic capacity. The control cells received the same handling- and PBS washing steps and received differentiation medium.

2.3.1 Treatment of cells with $AACOCF_3$ and Z-DEVD-FMK

Arachidonyl trifluoromethyl ketone ($AACOCF_3$) (10µM dissolved in ethanol), an inhibitor of $cPLA_2$ or Z-Asp (O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK) (10µM dissolved in DMSO), an inhibitor of caspase-3, -6, -7, -8, and -10 was added to the cells in differentiation medium for 30 min under normoxic conditions and 5% CO_2 followed by a 60 min washout phase in differentiation medium, prior to the simulated ischaemia.

2.3.2. Capase-3 silencing

Cells were transfected with 100 nM caspase-3 siRNA on day 6 of differentiation. 200 µl fresh differentiation medium was added to a microcentrifuge tube together with 4 µl transfection reagent and incubated at room temperature for 5 min. 12 µl caspase-3 siRNA was then added and gently mixed through pipetting up and down, followed by a 5 min incubation period at room temperature. The mixture was the added to 1000µl differentiation medium, which was added to the cells, followed by a 48 hour incubation period under normoxic conditions and 5% CO_2. Cells were then washed with fresh differentiation medium and submitted to the experimental protocol, where caspase-3 silenced cells were preconditioned with and without 0.5 ng/ml TNF-α. The caspase-3 silencing control cells received the same handling- and PBS washing steps and received differentiation medium.

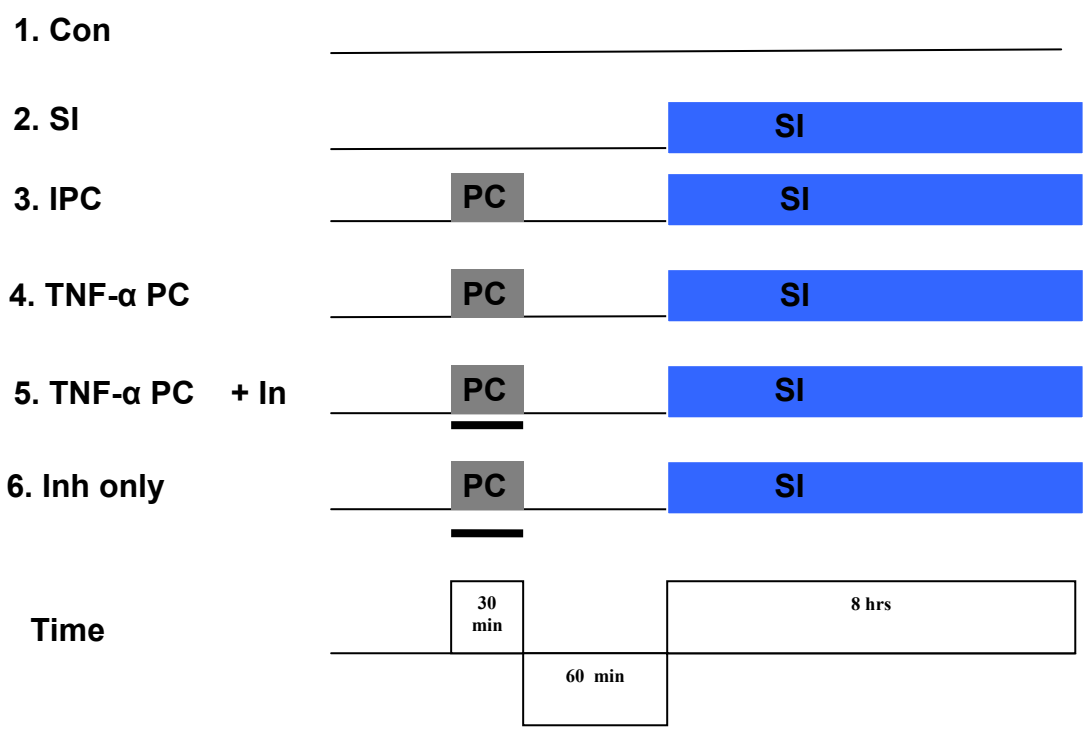


Fig.2.3. Schematic representation of experimental protocol with the groups: 1. control (Con), 2. simulated ischaemia (SI), 3. ischaemic preconditioning (IPC), 4. TNF-α preconditioning (TNF-α PC), 5. TNF-α + inhibitor (▬ AACOCF$_3$/Z-DEVD-FMK) and 6. inhibitor (Inh) only (AACOCF$_3$/Z-DEVD-FMK). SI lasted for 8 hrs. Ischaemic- or TNF-α preconditioning lasted for 30 min followed by a 60 min washout period.

2.4. Trypan blue viability assay

The trypan blue exclusion technique (Kitakaze *et al.*, 1997) was used to determine viability of C2C12 cells. Non-viable cells are unable to prevent the entrance of the blue dye into the cytoplasm due to membrane leakage, while it is excluded in viable cells due to an intact, functional cellular membrane. In brief, following simulated ischaemia, Esumi buffer was removed and cells were trypsinized through adding 0.25% trypsin, warmed up to 37ºC. Following a 3 min incubation time at 37ºC, cells were microscopically observed for detachment from the culture dish surface. To remove the trypsin and to neutralize the cell solution, cells were micro-centrifuged briefly at maximum speed, supernatant was taken off and cells were re-suspended in 100µl PBS. 100 µl trypan blue solution (4%) in PBS was added, and solutions were carefully mixed through re-suspension. Cells were immediately counted on the haemacytometer (Neubauer, Olympus, CKX31). The number of dark to total cells was calculated and viability expressed as a percentage of total cells counted.

2.5. Western blot analysis

2.5.1 Protein extraction

After completion of the experimental protocol, cells were rapidly placed on ice, Esumi buffer was removed and cells were carefully washed with cold PBS. Total myotube protein was extraceted with a lysis buffer (modified radioimmunoprecipitation (RIPA) buffer), pH 7.4, containing (in mM): Tris/HCl 2.5, ethylenediaminetetraacetic acid 1 (EDTA), NaF 50, NaPPi 50, dithiothreitol-1 (DTT), Phenylmethylsulfonyl fluoride (PMSF) 0.1, benzamidine 1,4 µg/ml soybean trypsin inhibitor (SBTI), 10 µg/ml leupeptin, 1% NP40, 0.1% SDS, 0.5% Na Deoxycholate. 260µl lysis buffer was added to the culture flask and cells were removed from the plastic surface using a cell scraper. Cell lysates were stored for short periods at -20°C in microcentrifuge tubes.

2.5.2. Bradford Protein quantitation

The rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Bradford, was employed (Bradford,

1976). It involves the binding of Coomassie Brilliant Blue G-250 to protein, which causes a shift in the absorption maximum of the dye from 465nm to 595 nm. The increase in absorption at 595nm is monitored, using a spectrophotometer (Cary Varian).

Cell lysates were defrosted, kept on ice and sonicated for 5 seconds at power level 3 (Vir Sonic 300, Virtis Gardiner) followed by centrifugation for 10 min at 4°C and 5000 x g (ALC-PK121R) in order to pellet cell debris and to expose the whole cellular protein fraction. For the establishment of a standard curve, a protein dilution series was set up, pipetting 2µg, 4µg, 8µg, 12µg, 12µg, 16µg and 20µg albumin bovine serum dissolved in PBS (BSA, 200µg/ml) and 900µl Bradford reagent into test tubes, adjusted to 1000µl with deionized water. Sample protein concentrations were determined through pipetting 5µl of the sample supernatant with 900µl Bradford reagent and adjusted to 1000µl with 95µl deionized water.

Test tube content was mixed by vortexing, incubated for 5 min at room temperature and the absorbance was measured at 595nm wavelength against a reagent blank prepared from 900 µl Bradford reagent and 100µl deionized water, using semi-micro cuvettes. The weight of protein in µg/ml was plotted against the absorbance and protein concentration was determined.

2.5.3. Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Laemmli sample buffer were prepared, using a sample buffer containing (in M): TRIS 0.5, pH 6.8, 10 % SDS, 2.5 ml glycerol, 0.2ml 0.5% bromophenol blue, deionized water. 150 µl mercaptoethanol was then added to 850 µl sample buffer. Cell lysates were boiled for 5 min and shortly spun in a microcentrifuge to assure complete sample acquisition. 20µg total protein was separated by 10% sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) and a 4% stack gel.

2.5.4. Western Blot analysis

After completion of the protein separation, proteins were transferred to a PVDF membrane (Immobilon^{TM} P, Millipore) using a semi dry blotting system (BIO RAD

trans-blot SD). 3 blotting buffers were prepared using for the anode buffer 1 (in M): Tris-base 0.3, pH 10.4, 20% methanol; for the anode buffer 2 (in mM): Tris-base 25, pH 10.4, 20% methanol and for the cathode buffer (in mM): Tris-base 25, ε-aminohexanoic acid 40, pH 9.4, 20% methanol. The PVDF membrane was soaked in methanol for 15 sec, allowed to dry and then soaked in anode buffer 2. On the anode plate four blotting papers were placed, after being soaked in anode buffer 1. Two blotting papers, soaked in anode buffer 2 were added, followed by the PVDF membrane and the gel, removed of its stack gel. Six blotting papers, soaked in cathode buffer, were added. To avoid air bubbles, the blotting sandwich was rolled with a clean wet glass tube. The system's cathode lid was tightly closed and the transfer proceeded with 0.5 A, 15 V for 60 min.

Following the protein transfer, non-specific binding sites on the membranes were blocked for 120 min at room temperature with 5% fat free milk in Tris-buffered saline-0.1% Tween 20 (Polyoxyethylenesorbitan monolaurate, Tween 20) (TBST). Followed by a washing step with TBST, the membranes were incubated overnight at 4°C with the primary antibodies that recognize phospho-specific $cPLA_2$ (Ser^{505}), ERK-p42/p44 (Thr^{202}/Tyr^{204}), p38-MAPK (Thr^{180}/Tyr^{182}), caspase-3 (p17 fragment pAb) and PARP (p85 fragment pAb). The primary antibody dilution was in each case 1:1000, diluted in TBST.

Pectora roborant cultus recti

Membranes were subsequently washed with large volumes of TBST (3x5 min) and the immobilized antibody conjugated with a 1:10 000 diluted horseradish peroxidase-labelled secondary antibody (Amersham Life Sciences), incubated for 50-60 min at room temperature. After thorough washing with TBST, membranes were incubated at room temperature for 1 min with ECL™ detection reagents (Amersham Life Sciences) and quickly exposed to an autoradiography film (Hyperfilm™) to detect light emission through a non-radioactive method (AXIM fixer/developer).

2.5.5. Film analysis

Films were densitometrically analysed (UN-SCAN-IT, Silkscience) and corrections were made for background noise.

2.6. Immunocytochemistry labelling

All solvents and buffers for the labelling were made up fresh and filter sterilized, to ensure that they are maximum particle free.

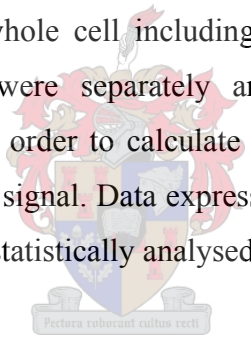
Cells were grown on autoclaved cover glasses which were placed into the culture dish. After completion of the experimental protocol 30 min into the ischaemic event, cells were gently washed with 0.1 M PBS and fixed using 1000 µl fixative per culture dish, made up as methanol/acetone in a 1:1 ratio. Culture dishes were incubated for 10 min on ice at 4˚C and then left to air-dry for 20 min at room temperature. Dried cover glasses were then gently rinsed with 1ml PBS and transferred to microscope slides.

For the rest of the labelling procedure slides were kept in light protected humidified staining chambers. The slides were then incubated at room temperature for 20 min with 5 % donkey serum in PBS, 100 µl per cover glass, to block non specific binding sites. Serum was then drained and primary antibody goat polyclonal nucleoporin p62 or goat polyclonal calnexin and rabbit polyclonal phospho-cPLA$_2$ (Ser^{505}) were added in a 1:50 PBS dilution and 50 µl volume and incubated for 90 min at room temperature. The application of the agents was performed in a manner that that a fluid bubble with a convex meniscus due to the fluid surface tension could occur without leaking of fluid from the cover glasses. For the controls for the staining procedure 50 µl PBS was used instead of primary antibody. The slides were then carefully washed with 1 ml PBS per slide and secondary antibody FITC or Texas Red (Jackson, donkey anti rabbit/donkey anti goat) was added in a 1:200 dilution and 50 µl volume to all slides, including the PBS controls. To avoid crystals, the secondary antibody was briefly centrifuged and the supernatant was used for the labelling. Following 30 min incubation at room temperature, 50 µl of the nuclear stain Hoechst, 10 mg/ml (Sigma) was added additionally in a 1:200 dilution and incubated for another 10 min at room temperature. Cover glasses were then carefully washed 3 times with 1000 µl PBS and transferred to glass microscope slides, mounted with fluorescent mounting medium (Dako Cytomation) and finally sealed with commercially available nail polish. Slides were then analysed and stored in silver foil at -20 ˚C.

2.6.1. Fluorescence intensity analysis

For the fluorescence image acquisition of the microscope slides, a Nikon eclipse E 400 microscope was used. Acquisition was performed under the same camera settings in terms of sensitivity and exposure time, using the Nikon ACT-1 software. The focal plane was selected using the Hoechst filter setting, to avoid unnecessary photo bleaching. P-cPLA$_2$ intensity was observed using the FITC filter setting. Images were taken with a 10x, 20x, 40x objective for documentation and with a 100x oil immersion objective for the intensity analysis, selecting 2 myotubes per field, for 6 random fields, in 4 independent experiments. Therefore, the data represent a total of 48 myotubes per experimental group.

Image analysis was performed employing the Simple PCI-C-Imaging Systems software. Images were loaded, and with the *Shapes Toolbar* the *region of interest* (ROI) tool was selected. The whole cell including the nuclei and the nuclei with overlayed cytoplasmic signal were separately analysed for mean fluorescence intensity and then subtracted, in order to calculate the mean nuclear intensity only, without cytoplasmic background signal. Data expressed in arbitrary pixel values were exported to Microsoft excel and statistically analysed.



2.6.2. z- stack analysis

In z-stack (3D) experiments stacks of images are acquired at different focal planes, which enable the construction of a three-dimensional image. This allows navigation through the image and the view in x, y and z dimensions separately in the *slice viewer*.

For the z stack analysis cells were grown in 8-chamber slides (Nunc) following the same labelling protocol as employed for the microscope slides. Image acquisition was performed using an Olympus IX 81 microscope equipped with the Cell^^{R} software. Through setting up a defined experiment in the *Experiment Manager*, image acquisition parameters such as exposure time, illumination settings and emission filter cube selection were kept constant for all groups and ensured appropriate selection of parameters. The DAPI 360 nm excitation wave length was used for setting the focal

plane, avoiding unnecessary photo-bleaching. For the experiment setting a combined *multi color image frame* was selected, using 360 nm DAPI for the nuclear stain Hoechst, 492 nm FITC for nucleoporin p62 or calnexin and 572 nm TxRed for the pcPLA$_2$ as multiple excitation settings. Through the *z-stack frame* the top and bottom focus position parameter were selected, indicating the upper and lower dimensions of the cell and a step width of 0.26 μm between the image levels was chosen.

2.6.3. Co-localization analysis

z-stack images were loaded into the image buffer and the two fluorescences to be measured were activated in the *image navigatior* button bar. Through the *co-localization* button bar the threshold for the first and second fluorescence was set. Co-localization was then calculated and displayed as a new false-color image. An Excel data sheet with co-localization area was generated.

2.7. Statistical analysis

The results are expressed as mean values ± SEM and were analyzed by one-way Analysis of Variance (ANOVA) with the Bonferroni correction. The α-level was 0.05. The statistics software Graph Pad Prism 2.01 was employed to perform statistical tests. Data were considered to be statistically significant with a p value <0.05.

Chapter 3: Results

3.1. Experimental measurements after 8 hrs simulated ischaemia

3.1.1. Cell viability after 8 hrs simulated ischaemia

To investigate the cytoprotective effect of 0.5 ng/ml TNF-α on C2C12 myotubes, cellular viability was assessed after the 8 hrs simulated ischaemic event, using the trypan blue exclusion method. As shown in Figure 3.1.1., control cells are highly viable (81.6±2.0%), whilst exposure to simulated ischaemia (SI) was potent enough to reduce viability significantly [24.8±3.9% ($p<0.05$)]. A significant increase in viability compared to SI was observed in cells exposed to IPC [57.6±3.7% ($p<0.05$)] and TNF-α PC [65.8±3.4% ($p<0.05$)].

In order to study the involvement of $cPLA_2$ in the protection conferred by TNF-α we used $AACOCF_3$ to inhibit $cPLA_2$. We undertook investigations to reveal the involvement of $cPLA_2$ in TNF-α mediated cytoprotection, using the C2C12 cell line, a murine skeletal muscle cell line as model for cardiac tissue, based on previous work of peers as well as on findings described in skeletal muscle preconditioning studies performed by Pang *et al* (1996). The C2C12 model is an established and recognized model for cardiac and skeletal muscle tissue.

The inhibitor conferred cytoprotection significantly [52.8±1.9% ($p<0.05$)] with viability values close to those seen in the IPC group. The inhibitor applied together with 0.5 ng/ml TNF-α also conferred cytoprotection [53.7±1.1% ($p<0.05$)], with values similar to those seen when using the $cPLA_2$ inhibitor only. The vehicle effect was ruled out, since appropriate experiments with the solvent, ethanol, did not show any effect.

3.1.2. $cPLA_2$ phosphorylation after 8 hrs simulated ischaemia

The phosphorylation pattern of $cPLA_2$ following 8 hrs simulated ischaemia was assessed using Western blot analysis, reflecting the whole cell protein fraction. Data are expressed as % of control (Figure 3.1.2.). Compared to the control phosphorylation level, a significant downregulation in $cPLA_2$ phosphorylation in IPC

[20.3±5.2% ($p<0.05$)] and TNF-α PC [45.3±5.0% ($p<0.05$)] can be observed. In SI $cPLA_2$ phosphorylation is similarly decreased [41.1±0.5% ($p<0.05$)].

To assess a possible modulatory effect of caspase-3 on $cPLA_2$ phosphorylation or $cPLA_2$ cleavage, the caspase inhibitor Z-DEVD-FMK (10μM) was employed. Under the effect of this inhibitor, $cPLA_2$ phosphorylation was almost completely inhibited, with significantly less $cPLA_2$ phosphorylation [3.7±0.7% ($p<0.05$)] compared with the TNF-α PC group. This inhibitory effect however was overwritten, when using Z-DEVD-FMK in the presence of 0.5 ng/ml TNF-α [35.2±0.5 ($p<0.05$)].

3.1.3. Caspase-3- and PARP cleavage after 8 hrs simulated ischaemia

In order to investigate the recruitment of the apoptotic pathway after 8 hrs simulated ischaemia, caspase-3 cleavage (Figure 3.1.3.) and PARP cleavage (Figure 3.1.4.) were assessed through Western blot analysis. Figure 3.1.3. indicates clearly, that compared to control levels, caspase-3 is cleaved showing the 17 kDa cleaved product, reaching significance in the SI group [194.8±7.9% ($p<0.05$)] as well as in the TNF-α PC group [229.0±13.2% $p<0.05$)]. The caspase inhibitor applied in the presence of 0.5 ng/ml TNF-α significantly increases caspase-3 cleavage [339.2±15.6% $p<0.05$)] compared to TNF-α PC only. The caspase inhibitor reduces caspase-3 cleavage [99.1±18.6] towards the control level, indicating its physiological activity.

The 116 kDa PARP protein levels were analyzed. The less dense this band the more PARP has been cleaved, reducing the 116 kDa signal intensity. Compared to the PARP control level significant decreases in total PARP in the SI [43.8±1.0% ($p<0.05$)], IPC [34.9±3.1% ($p<0.05$)] and TNF-α PC groups [33.7±2.6% $p<0.05$)] were observed. The caspase inhibitor induced PARP cleavage, however, significantly less than seen in the previous 3 groups [71.2±3.9% ($p<0.05$)]. When the caspase inhibitor is employed in the presence of 0.5 ng/ml TNF-α, significantly less total PARP is observed [56.9±8.7% ($p<0.05$)] compared to the PARP signal seen with the caspase inhibitor only; however, it is a significantly stronger PARP signal in the TNF-α + Inh (Z-DEVD-FMK) group compared with SI, IPC and TNF-α PC.

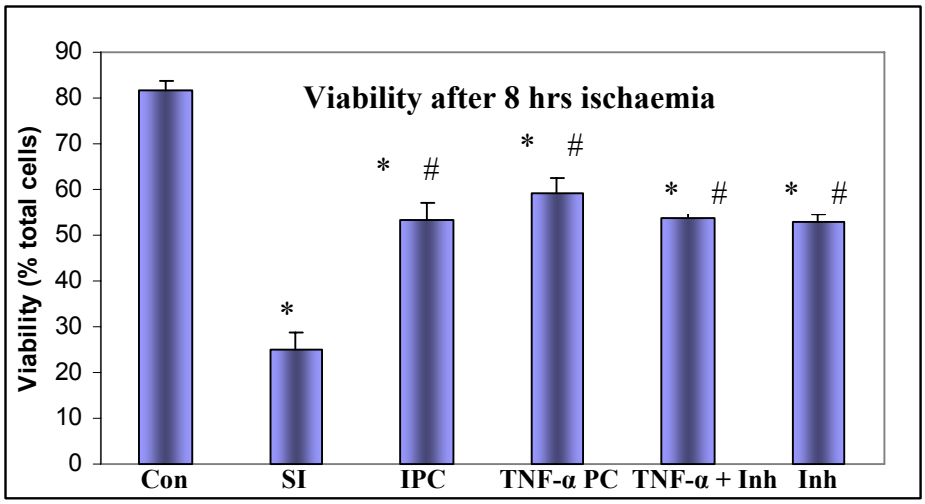


Figure 3.1.1. Viability in % following simulated ischaemia showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh and inhibitor only. SI lasted for 8 hrs. Ischaemic- or TNF-α preconditioning lasted for 30 min followed by a 60 min washout period. Inh=AACOCF$_3$. * $p<0.05$ vs Con, # $p<0.05$ vs SI, n=6.

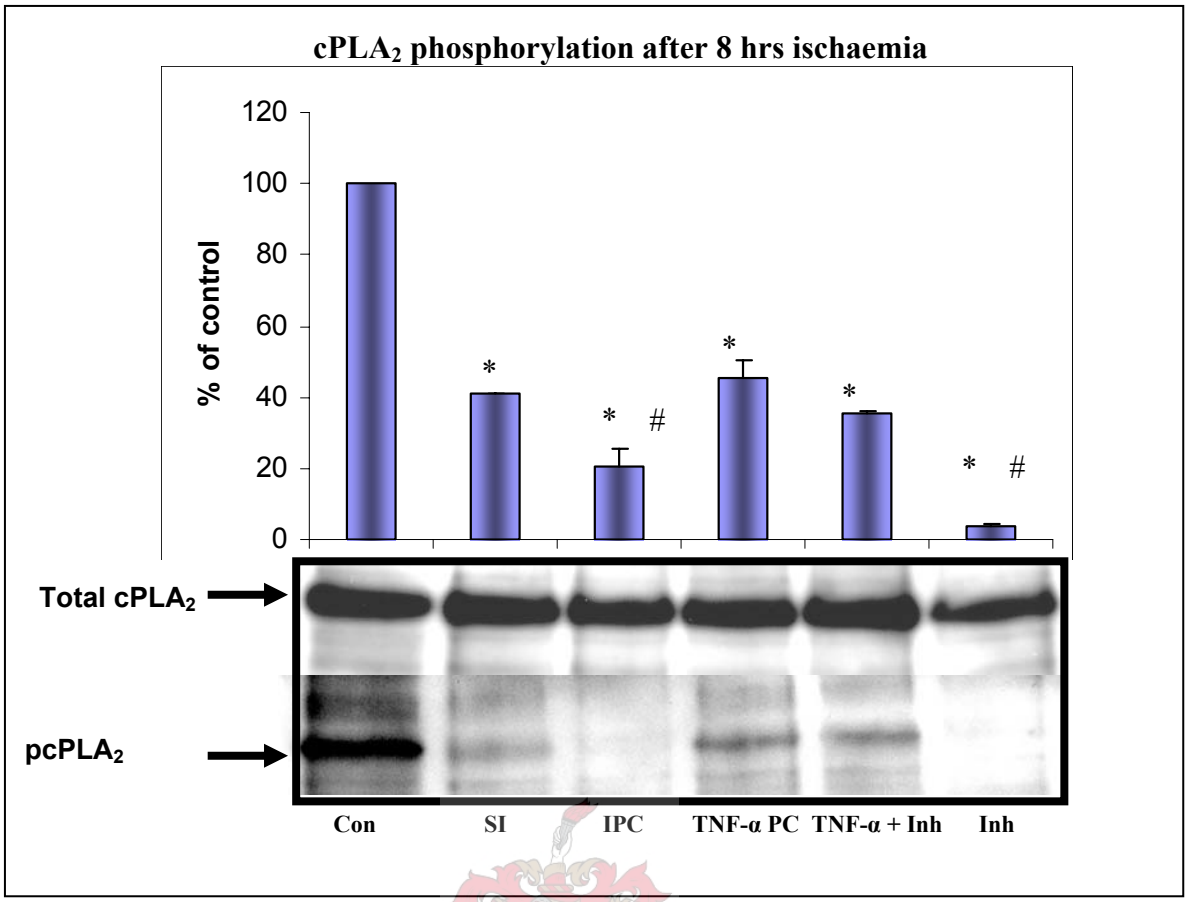


Figure 3.1.2. $cPLA_2$ phosphorylation following simulated ischaemia showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh and inhibitor (Inh) only. SI lasted for 8 hrs. Inh= Z-DEVD-FMK. * $p<0.05$ vs Con, # $p<0.05$ vs SI, n=6.

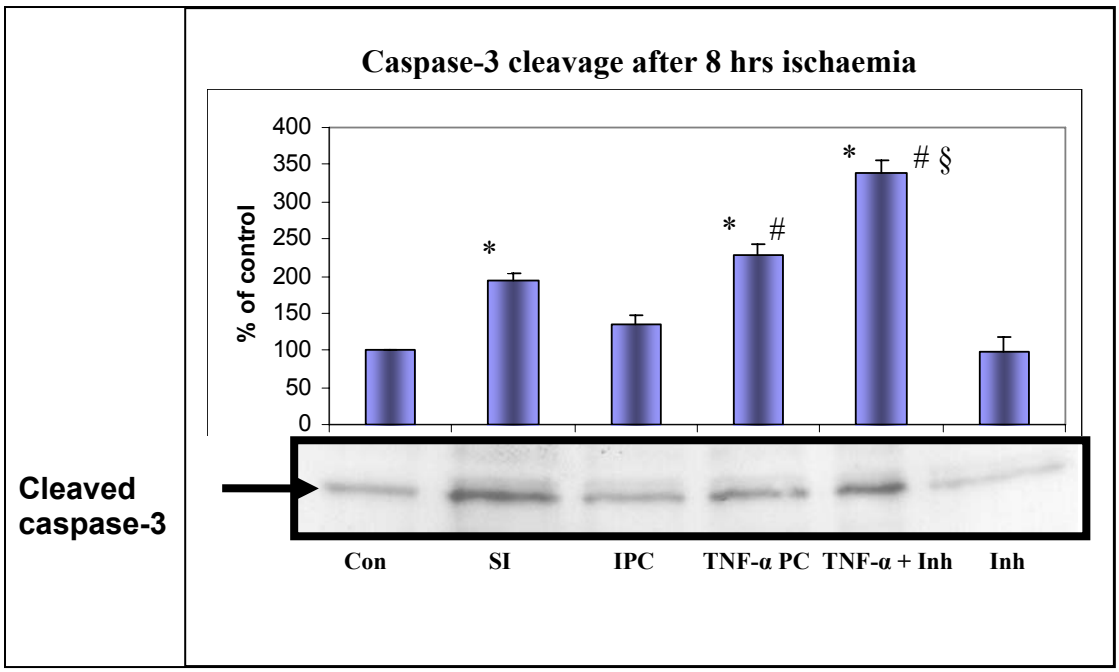


Figure 3.1.3. Caspase-3 cleavage indicated through the 17 kDa fragment following simulated ischaemia showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh and inhibitor (Inh) only. SI lasted for 8 hrs. Inh = Z-DEVD-FMK. * $p<0.05$ vs Con, # $p<0.05$ vs IPC, § $p<0.05$ vs TNF-α PC, n=6.

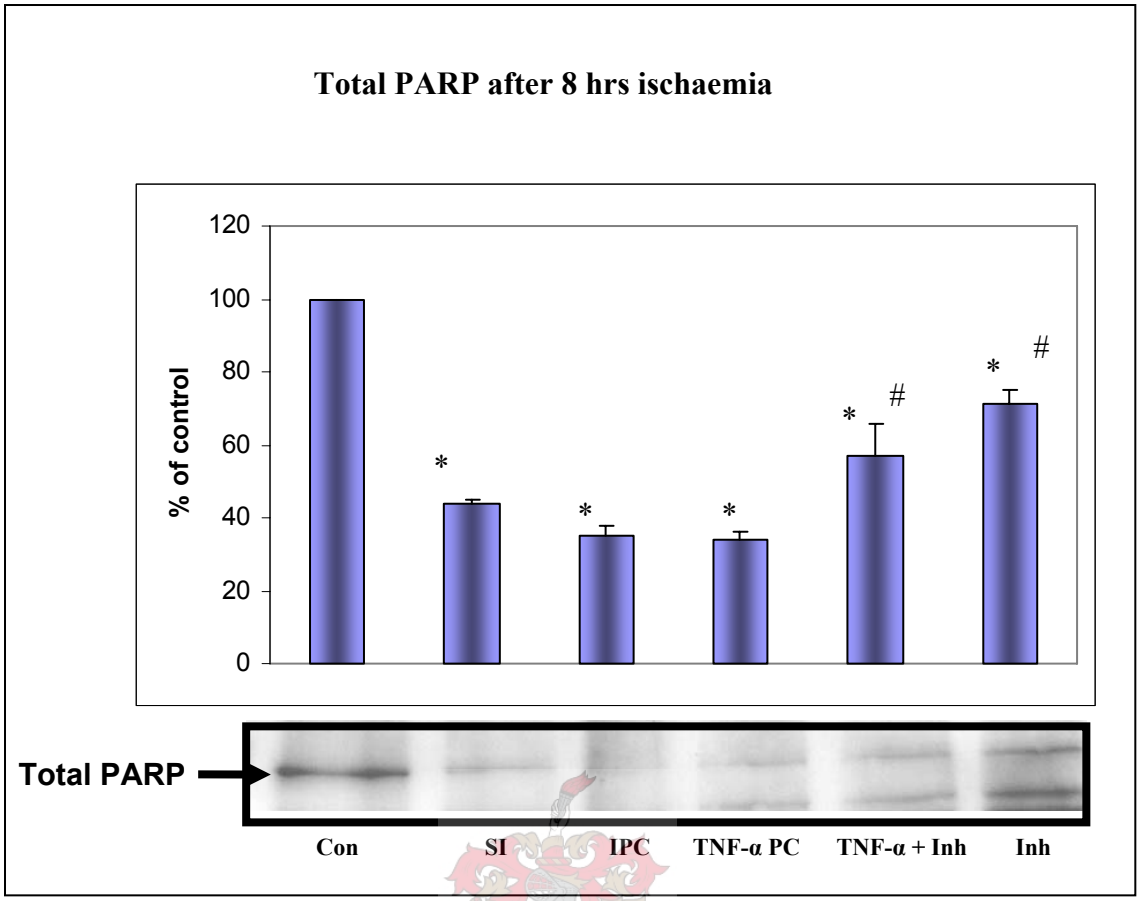


Figure 3.1.4. PARP cleavage indicated through the 116 kDa fragment following simulated ischaemia showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh and inhibitor (Inh) only. SI lasted for 8 hrs. Inh= Z-DEVD-FMK.* $p<0.05$ vs Con, # $p<0.05$ vs IPC, n=6.

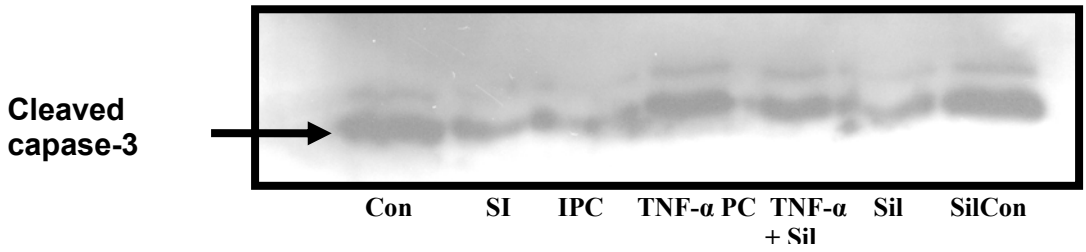


Figure 3.1.5. Non-successful caspase-3 silencing. Caspase-3 cleavage indicated through the 17 kDa fragment following simulated ischaemia showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + caspase-3 silencing (TNF-α + Sil), caspase-3 silencing only (Sil) and caspase-3 silencing control (SilCon). SI lasted for 8 hrs, n=1.

3.2. 5 min into simulated ischaemia

3.2.1. $cPLA_2$ phosphorylation at 5 min into the simulated ischaemic event

At 5 min into the ischaemic event, the $cPLA_2$ phosphorylation pattern was investigated. In order to find a cross talk between caspase-3 and the degree of $cPLA_2$ phosphorylation, the caspase inhibitor Z-DEVD-FMK was employed. Compared to the control levels, no statistically siginificant differences were observed (Figure 3.2.1.). Since the phosphorylation band was very weak in all groups, the effect of the $cPLA_2$ inhibitor could not be evaluated.

3.2.2. ERK and p38 phosphorylation at 5 min into the simulated ischaemic event

It is suggested in the literature that both p38 MAPK and ERK can phosphorylate $cPLA_2$. We therefore investigated the phosphorylation pattern of both MAPK at 5 min into the ischaemic event. To elucidate the $cPLA_2$ activation pathway in context of TNF-α preconditioning, we employed a caspase inhibitor (Z-DEVD-FMK) and a $cPLA_2$ inhibitor ($AACOCF_3$) in the presence of 0.5 ng/ml TNF-α as well as the inhibitor only. However, at the chosen time point no significant changes in the ERK phosphorylation pattern were observed (Figure 3.2.2.a.). When investigating p38 phosphorylation we found a weak pp38 signal in the control group; however the phosphorylation did not reach significance compared with the other groups (Figure 3.2.2.b.). However we did find a significant increase in p38 phosphorylation when employing the caspase inhibitor alone [96.2±13.4% ($p<0.05$)] or in presence of 0.5 ng/ml TNF-α [102.3±22.4% ($p<0.05$)] compared to the pp38 signal observed when employing the $cPLA_2$ inhibitor in presence of 0.5 ng/ml TNF-α.

3.2.3 Caspase-3- and PARP cleavage at 5 min into the simulated ischaemic event

The observation of caspase-3 or PARP cleavage early within the ischaemic event enables us to dissect out the onset of the apoptotic pathway induction. As can be seen in Figure 3.2.3.a. we can observe a very interesting trend in caspase-3 cleavage, which however does not reach statistical significance at that time point. The extent of caspase-3 cleavage in the Con and SI group is very small, indicated by a faint band of the 17 kDa cleaved caspase-3 unit. However, we find a trend towards caspase-3

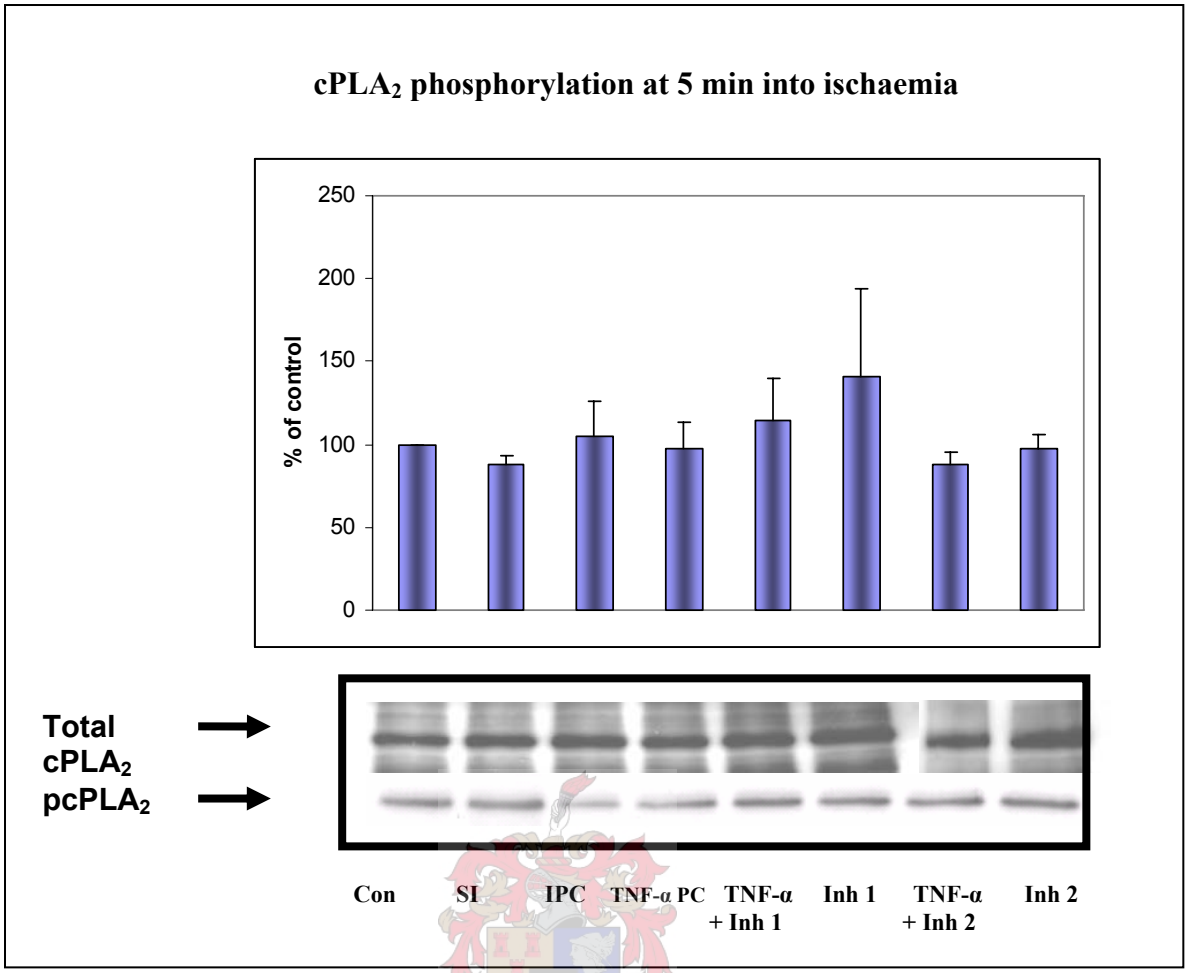


Figure 3.2.1. $cPLA_2$ phosphorylation at 5 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No significant changes were observed.

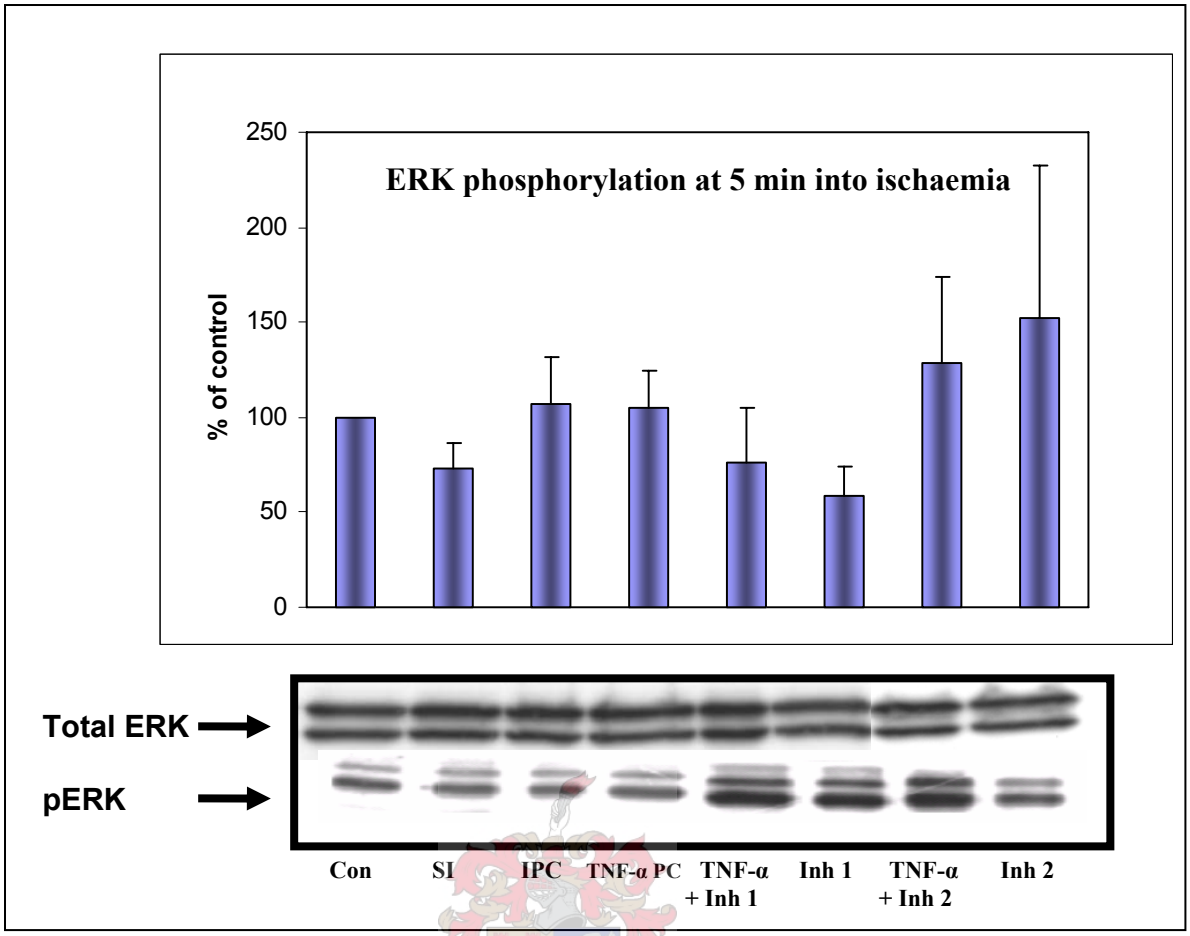


Figure 3.2.2.a. ERK phosphorylation at 5 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No phosphorylation of ERK was observed.

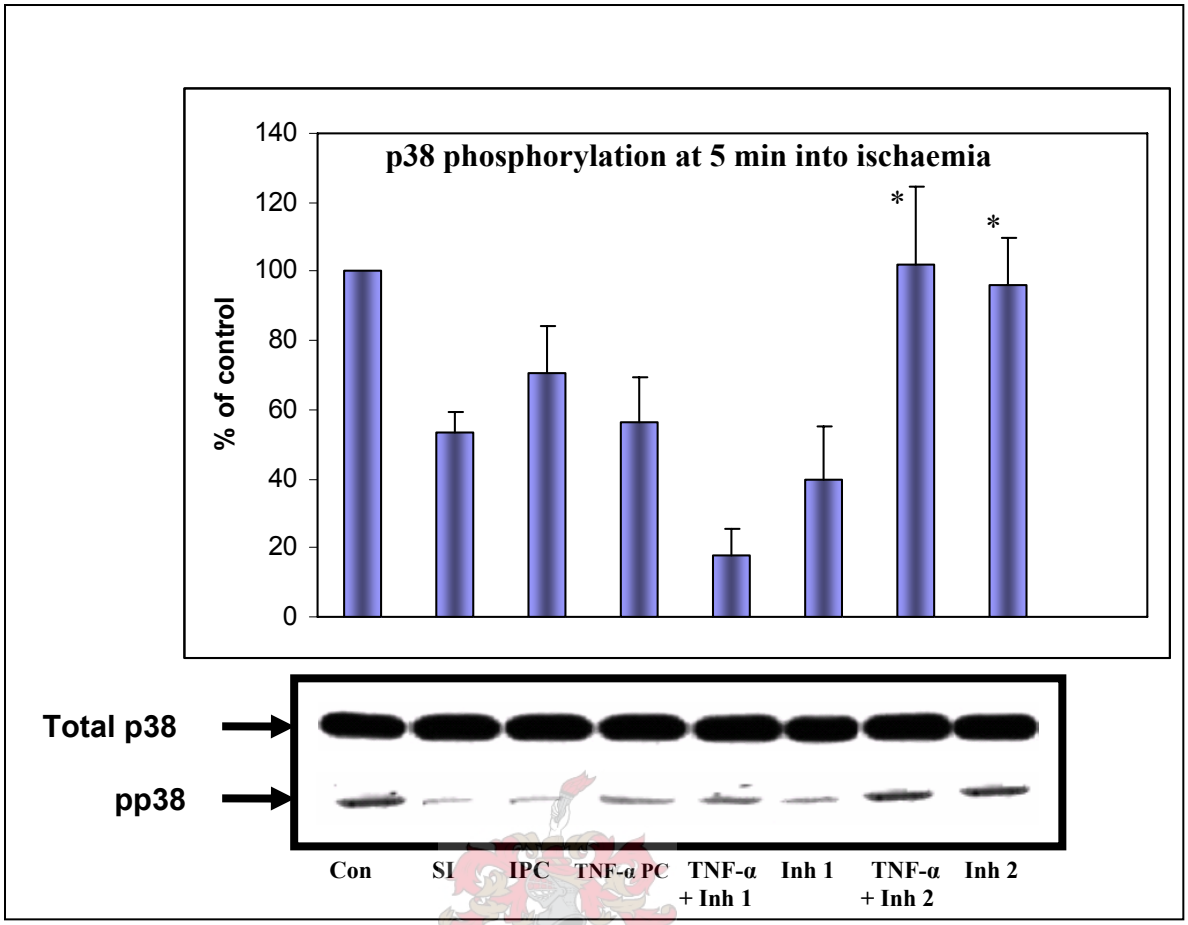


Figure 3.2.2.b. p38 phosphorylation at 5 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. * $p<0.05$ vs TNF-α + Inh 1. (The graph is a reflection of all conducted experiments, which is not in all cases coherently reflected in the western blot image).

cleavage in both the IPC and TNF-α PC group. The caspase-3 inhibitor did not affect caspase-3 cleavage yet, indicated by a much stronger signal. When looking at PARP cleavage at the same time point (Figure 3.2.3.b.), we find no significant differences between the groups, indicating that PARP cleavage is not executed that early in ischaemia. This is to be expected, since caspase-3 cleavage is a prerequisite for PARP cleavage to take place.

3.3. 30 min into simulated ischaemia

3.3.1. $cPLA_2$ phosphorylation at 30 min into the simulated ischaemic event

A second appropriate time window in the ischaemic event was chosen, to investigate $cPLA_2$ phosphorylation- and activity changes. Even at 30 min into simulated ischaemia, no significant changes of $cPLA_2$ phosphorylation were observed (Figure 3.3.1.).

3.3.2. ERK and p38 phosphorylation at 30 min into the simulated ischaemic event

The phosphorylation of p38 and ERK MAPK was investigated at 30 min into simulated ischaemia. As shown in Figure 3.3.2.a., a significant increase in ERK phosphorylation can be observed when using $AACOCF_3$ alone [283.2±30.8% ($p<0.05$)] or in presence of 0.5 ng/ml TNF-α [312.9±31.3% ($p<0.05$)], compared to control levels. This also reaches significance when compared to the SI group. The caspase inhibitor Z-DEVD-FMK alone [46.9±16.1%] or in presence of 0.5 ng/ml TNF-α [55.1±21.5%] prevents ERK phosphorylation, keeping the level similar to control levels. This reaches significance when comparing with the groups using $AACOCF_3$ alone or in presence of 0.5 ng/ml TNF-α.

When looking at p38 phosphorylation, we find a significant increase in the IPC group compared to SI (Figure 3.3.2.b.). This increase in phosphorylation is however not statistically significant when comparing to control levels. The increase in p38 phosphorylation in IPC also reaches significance when comparing with TNF-α + Inh 1- or 2; and Inh 1- or 2 only.

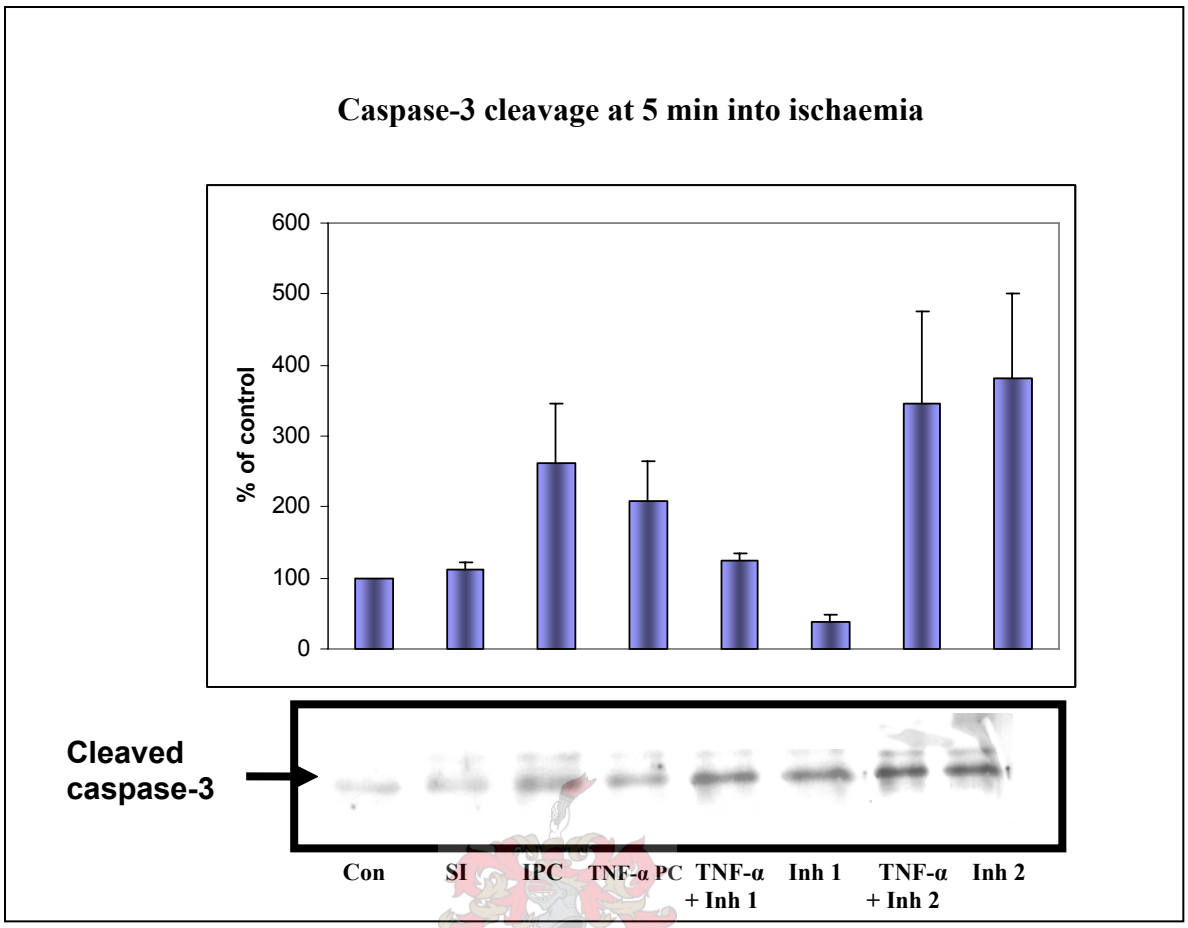


Figure 3.2.3.a. Caspase-3 cleavage at 5 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No significant changes were observed.

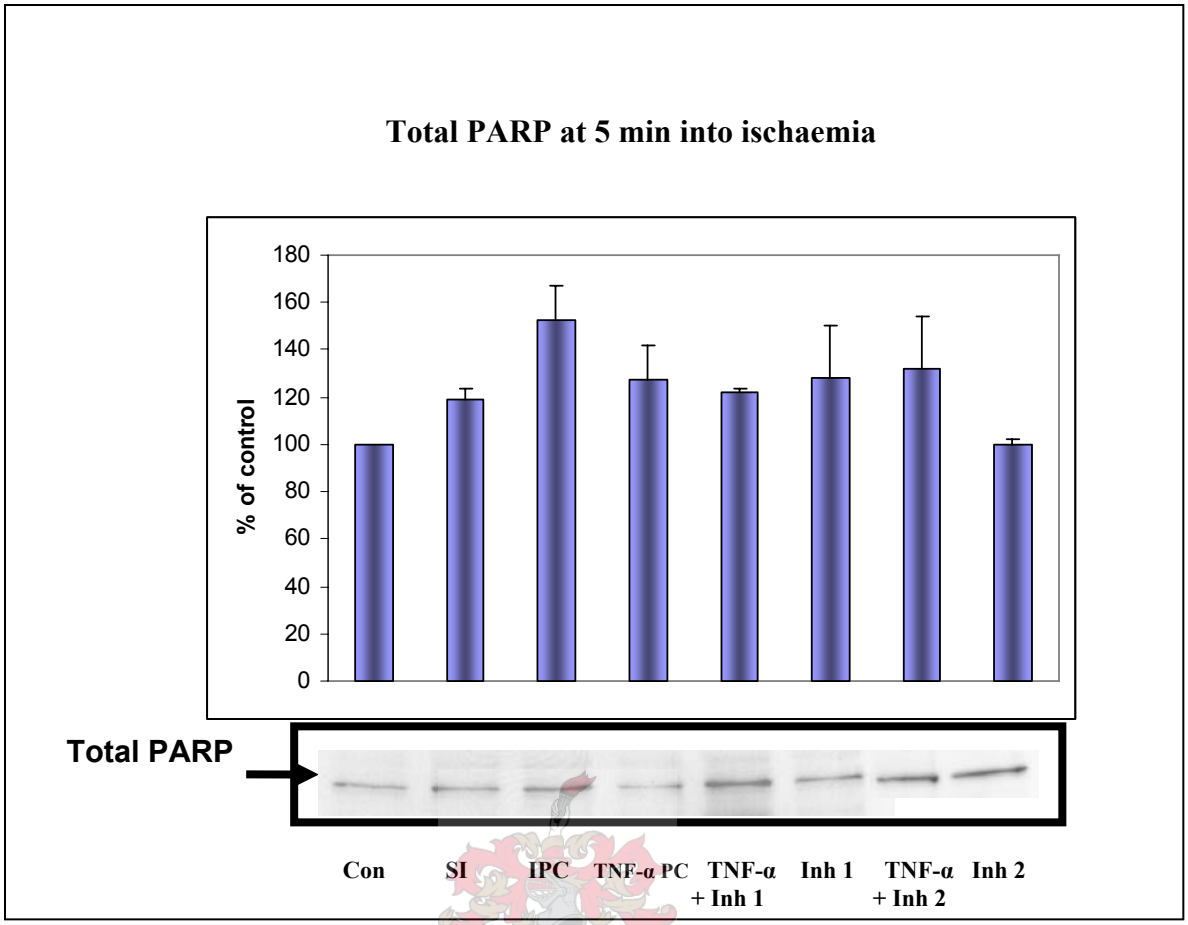


Figure 3.2.3.b. Total PARP at 5 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No significant changes were observed.

3.3.3. Caspase-3- and PARP cleavage at 30 min into the simulated ischaemic event

The trend of caspase-3 cleavage as seen at the 5 min time point, is clearly mirrored at the 30 min time point into the ischaemic event (Figure 3.3.3..a). However, no statistical significance is reached. The trend displays an increase in caspase-3 cleavage in the order SI, IPC and TNF-α PC. A clear decrease in caspase-3 cleavage is seen when employing the $cPLA_2$ inhibitor $AACOCF_3$ alone or in presence of 0.5 ng/ml TNF-α. Subsequently, we do not find any significant changes in the PARP cleavage pattern, as reflected by a similar strong signal of the 116 kDa band seen in all groups (Figure 3.3.3.b.).

3.4. Vehicle controls

To verify that there was no effect of the solvents ethanol and DMSO used for the $cPLA_2$ inhibitor $AACOCF_3$ and the caspase inhibitor Z-DEVD-FMK respectively, phosphorylation- and cleavage pattern of the proteins of interest were assessed. Figure 3.4. shows that a vehicle effect could be ruled out.

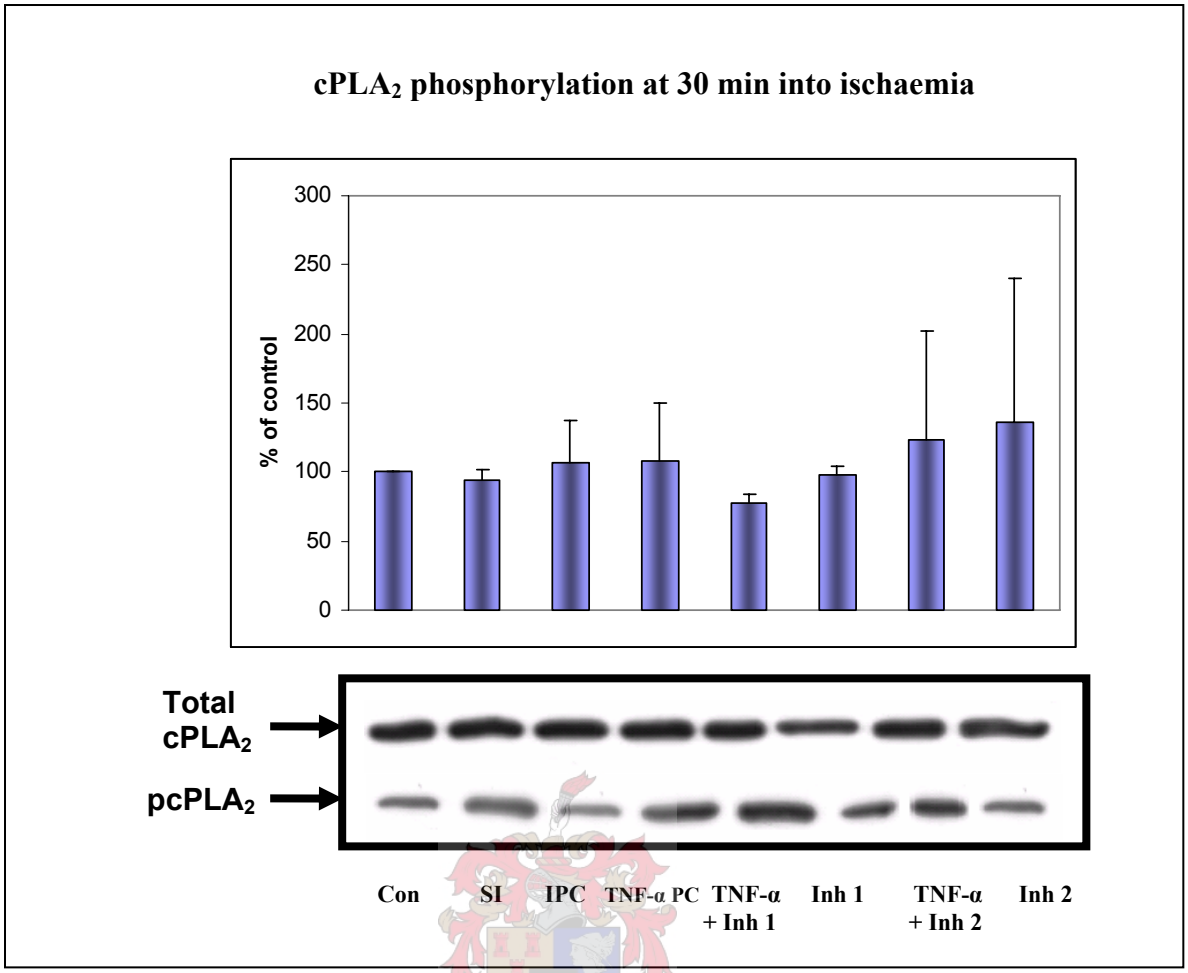


Figure 3.3.1. $cPLA_2$ phosphorylation at 30 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No significant changes were observed.

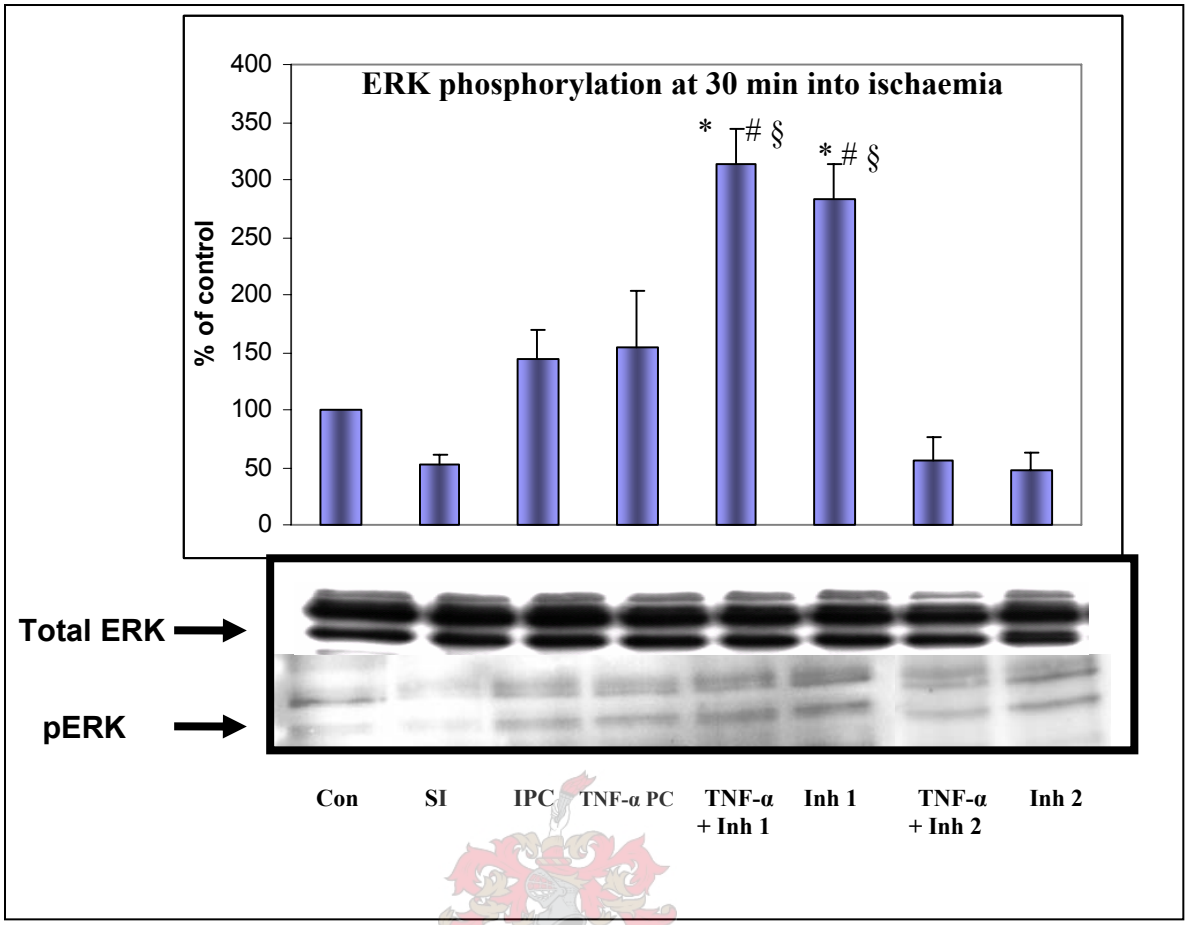


Figure 3.3.2.a. ERK phosphorylation at 30 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. * $p<0.05$ vs Con, # $p<0.05$ vs SI, § $p<0.05$ vs TNF-α + Inh 2 and Inh 2 only.

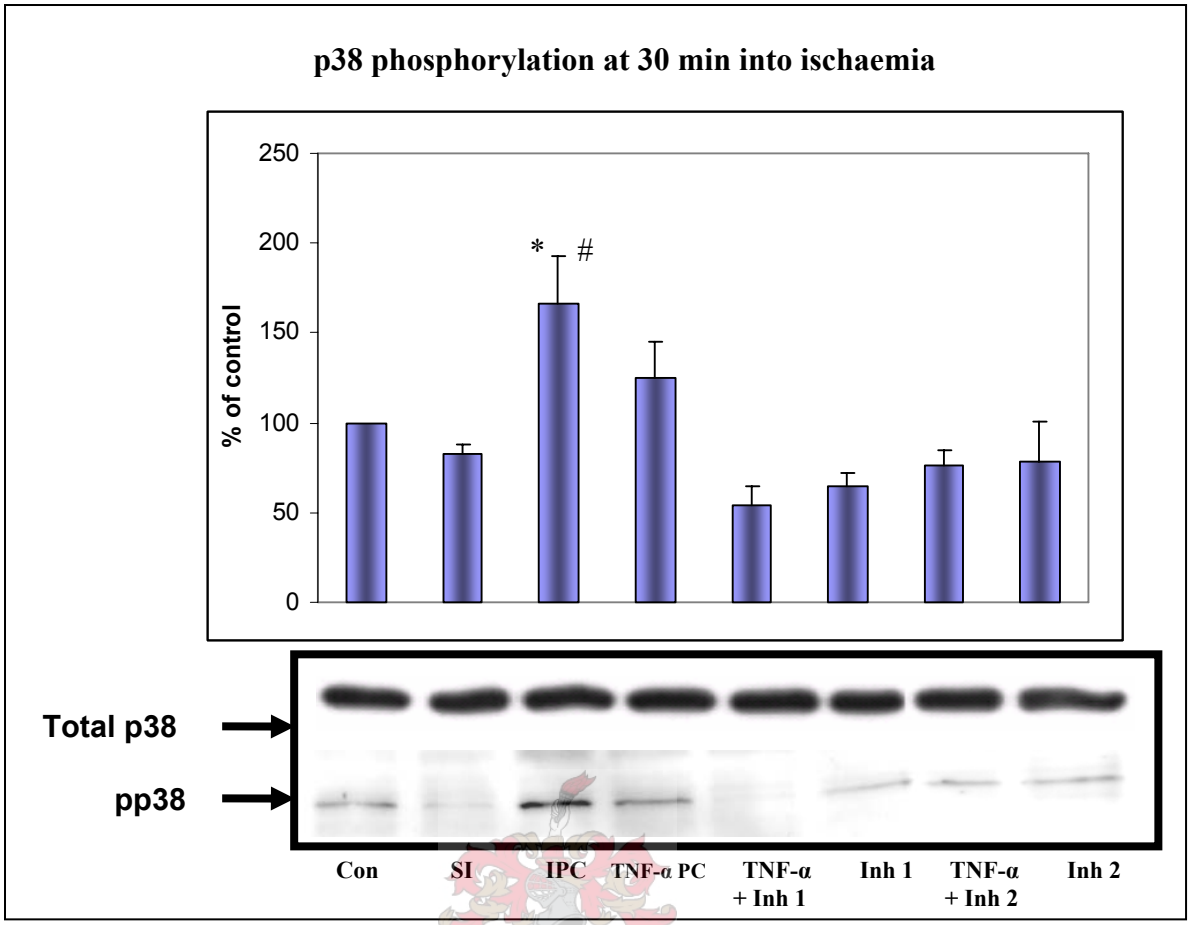


Figure 3.3.2.b. p38 phosphorylation at 30 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. * $p<0.05$ vs SI, # $p<0.05$ vs TNF-α + Inh 1- and 2; and Inh 1- and 2 only.

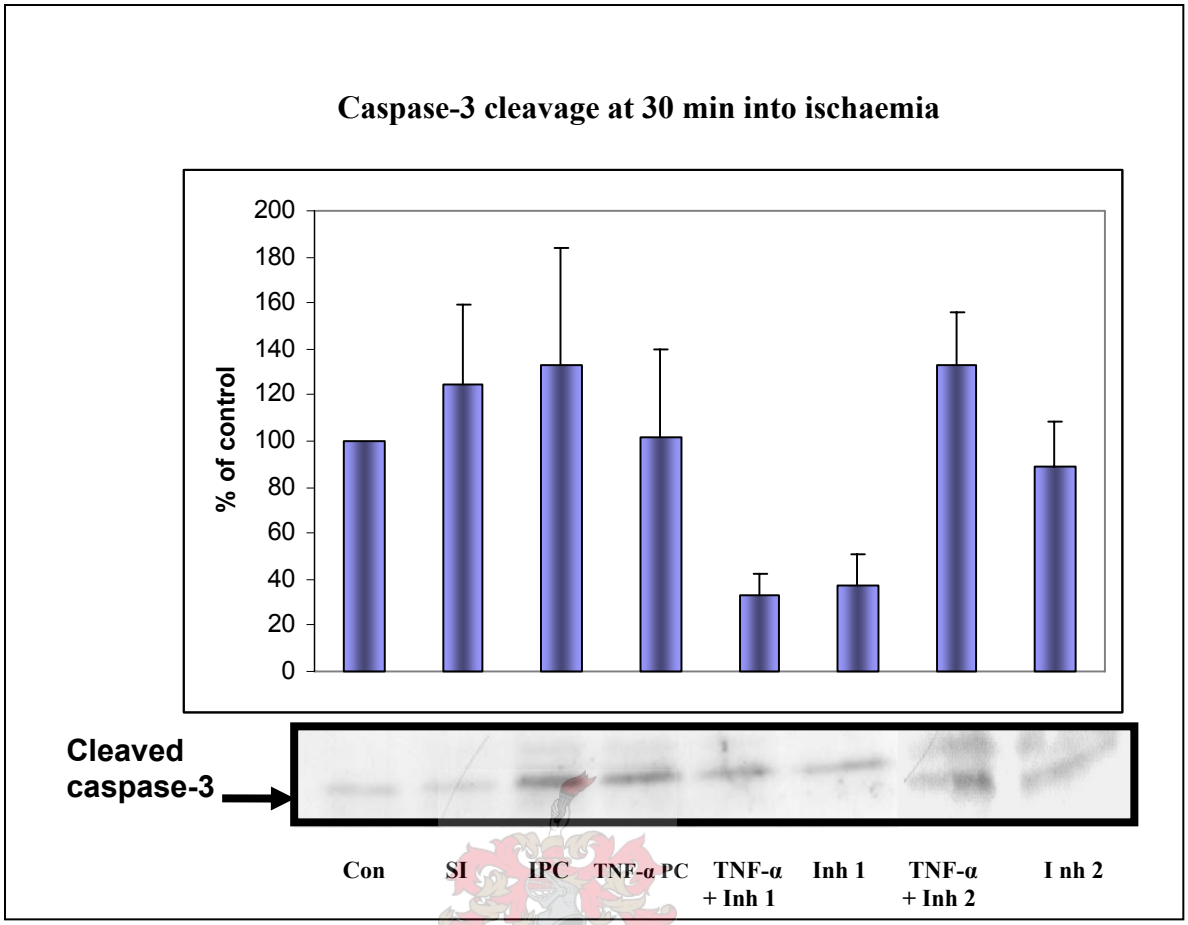


Figure 3.3.3.a. Caspase-3 cleavage at 30 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. Although a trend has been observed, no significant changes were present.

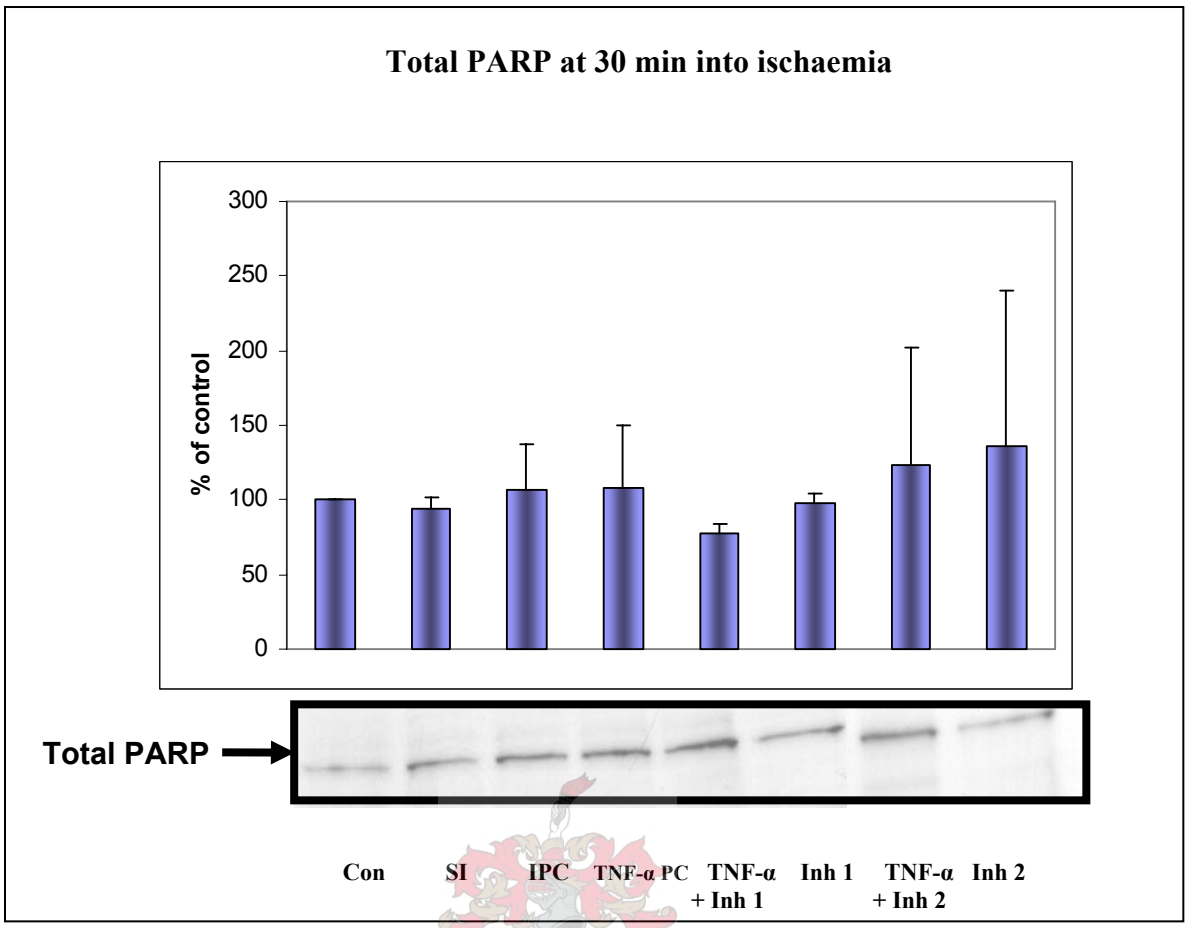


Figure 3.3.3.b. Total PARP at 30 min into the ischaemic event, showing control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC), TNF-α preconditioning (TNF-α PC), TNF-α + Inh 1 and inhibitor 1 (Inh 1) only as well as TNF-α + Inh 2 and inhibitor 2 (Inh 2) only. Inh 1 = $AACOCF_3$, Inh 2 = Z-DEVD-FMK, n=6. No significant changes were observed.

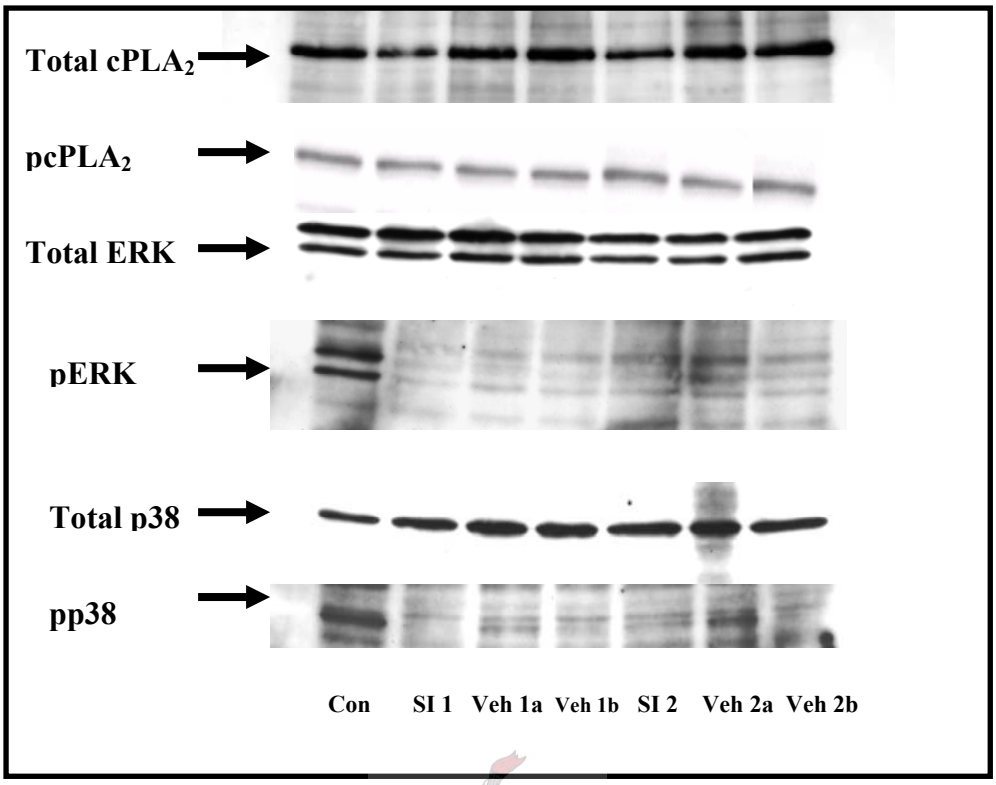


Figure 3.4. Vehicle controls for the proteins of interest, showing the control group (Con), simulated ischaemia at 5 min (SI 1), Z-DEVD-FMK vehicle (DMSO) at 5 min ischaemia (Veh 1a), $AACOCF_3$ vechile (ethanol) at 5 min ischaemia (Veh 1b), simulated ischaemia at 30 min (SI 2), Z-DEVD-FMK vehicle (DMSO) at 30 min ischaemia (Veh 2a) and $AACOCF_3$ vehicle (ethanol) at 30 min ischaemia (Veh 2b). No effects of the vehicle on the phosphorylation- and cleavage pattern were observed.

3.5. Immunocytochemistry

In order to investigate the translocation pattern of phosphorylated $cPLA_2$, immunocytochemistry was performed. A high magnification (100 x oil immersion) was chosen, to enable the identification of subcellular regions involved in $pcPLA_2$ signalling events. The nuclear stain HOECHST was employed to indicate the nuclear region.

3.5.1. $cPLA_2$ phosphorylation and mean nuclear intensity at 30 min into the simulated ischaemic event

Figure 3.5.1.a. and 3.5.1.b. clearly indicate a differential $pcPLA_2$ signal in the nuclear region in the different groups, indicated with the HOECHST nuclear stain. Without performing any analysis, the SI myotubes appeared to show a very strong nuclear $pcPLA_2$ signal, compared to the control group. TNF-α PC displayed a weak nuclear signal, similar to the control group. IPC displayed a stronger nuclear signal, but weaker then the SI group.

Via the selection of a region of interest (ROI), the mean nuclear intensity was compared (Figure 3.5.c). Results verify the nuclear intensity profile perceived with the "unarmed" eye. SI showed a significant increase in mean nuclear intensity [40.6±5.4 ($p<0.05$)], compared with the control group [15.9±2.2]. In IPC [27.3±1.3 ($p<0.05$)] and TNF-α PC [25.4±3.2 ($p<0.05$)] significantly less nuclear intensity was observed, compared to the SI group.

3.5.2. $cPLA_2$ phosphorylation and 3 D z-stack fluorescence imaging at 30 min into the simulated ischaemic event

The z-stack technique enabled the construction of a 3 dimensional myotube image, to observe $pcPLA_2$ translocation more in depth. As seen in Figure 3.5.2.a. and 3.5.2.b., the $pcPLA_2$ translocation pattern in all four groups mirrors the results described using the other labelling and image acquisition techniques (Figure 3.5.1.a. and 3.5.1.b.). Maximum nuclear intensity is seen in SI, whilst TNF-α PC shows fluorescence intensity close to the control levels. Furthermore, it was observed that $pcPLA_2$ translocates to the peri- and endonuclear region. It also seems that IPC shows a stronger nuclear signal than TNF-α PC; however, as seen in Figure 3.5.1.c., this does not even reach statistical significance compared to the Con group intensity.

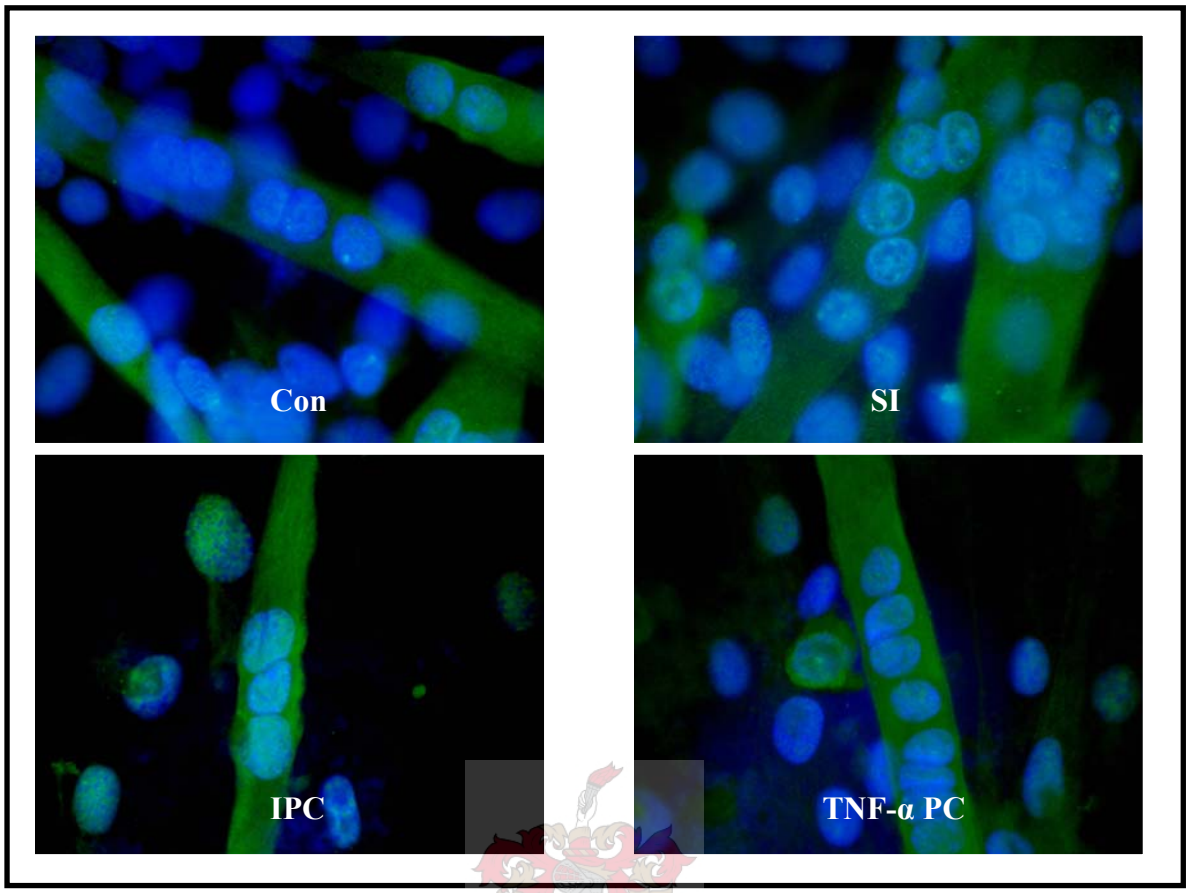


Figure 3.5.1.a. C2C12 myotubes labeled with pcPLA2/FITC, displayed in green and the nuclear indicator HOECHST, displayed in blue. The figure shows control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC).

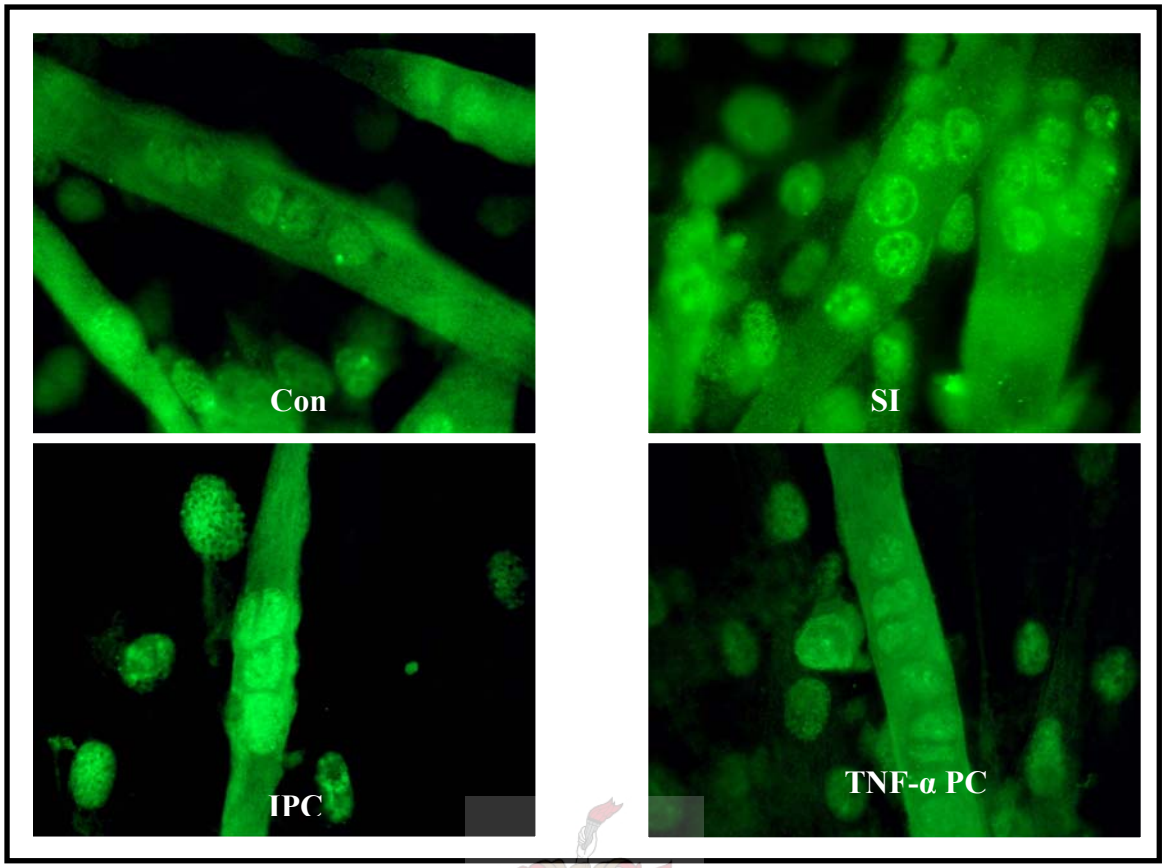


Figure 3.5.1.b C2C12 myotubes labeled with pcPLA2/FITC only. The figure shows control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC).

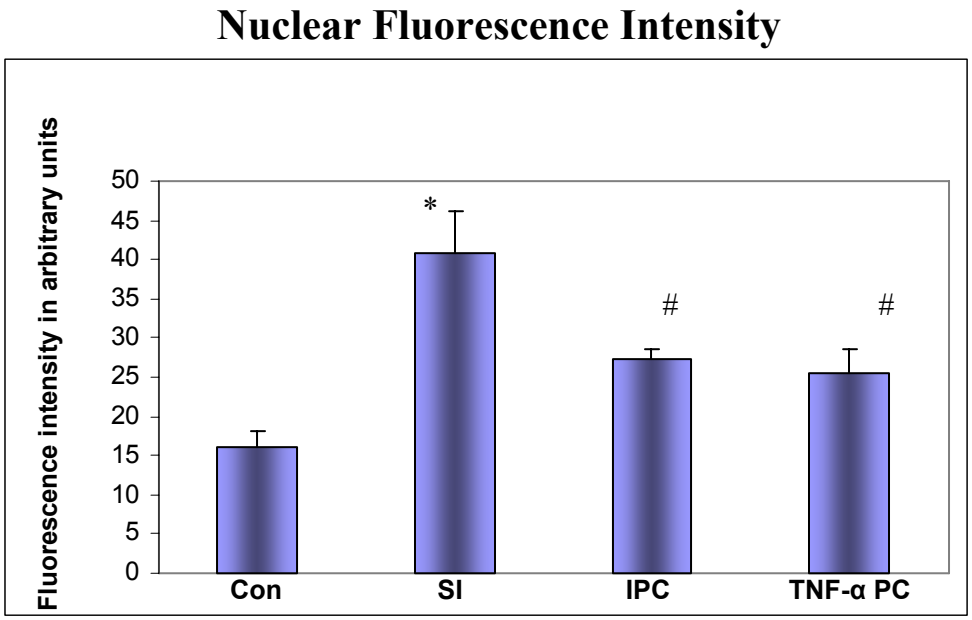


Figure 3.5.1.c Mean $cPLA_2$ fluorescence nuclear intensity is shown in control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC). * $p<0.05$ vs Con, # $p<0.05$ vs SI, n=4.

3.5.3. Co-localization of pcPLA$_2$ and nucleoporin p62, 3 D z-stack imaging at 30 min into the simulated ischaemic event

In order to verify nuclear translocation of pcPLA$_2$, a nuclear marker protein, nucleoporin p62 was chosen (Figure 3.5.3.a.) together with the co-localization technique. Cells were co-labelled for nucleoporin p62 and pcPLA$_2$, a z-stack was acquired with a step width of 0.26 µm and co-localization area was determined by the software, generating a new image (Figure 3.5.3.b.). The results (Figure 3.5.3.c.) show a significant increase in co-localization area in the SI group [5.8±0.3% ($p<0.05$)] compared to control levels. Furthermore, a significantly reduced co-localization area is observed in IPC [2.2±0.3% ($p<0.05$)] and TNF-α PC [1.7±0.2% ($p<0.05$)] compared to the area in the SI group.

3.5.4. Differential pcPLA$_2$ translocation to endoplasmic reticulum (ER) in SI?

When carefully observing Figure 3.5.1.b., a clustered or punctuate fluorescence signal pattern can be seen in the SI group, representing pcPLA$_2$. To investigate whether SI induces a differential translocation response, possibly involving the endoplasmic reticulum (ER) as target organelle, labelling for the ER was performed. The marker protein for this organelle, calnexin, was chosen. The image (Figure 3.5.4.) indicates a similar punctuate or cluster fluorescence signal pattern for calnexin, as seen in SI for pcPLA$_2$. This provides preliminary evidence for a differential organelle targeting mechanism in SI, including not only the nucleus but also the ER.

3.6. DVD

Due to the nature of the acquired data as well as space limiations, a data DVD is attached with accompanying image and movie clip material. The DVD contains z-stack movies of pcPLA$_2$, pcPLA$_2$ and nucleoporin; and pcPLA$_2$ and calnexin; as well as the co-localization z-stacks showing of each the control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC). Furthermore the PBS-control stains are included.

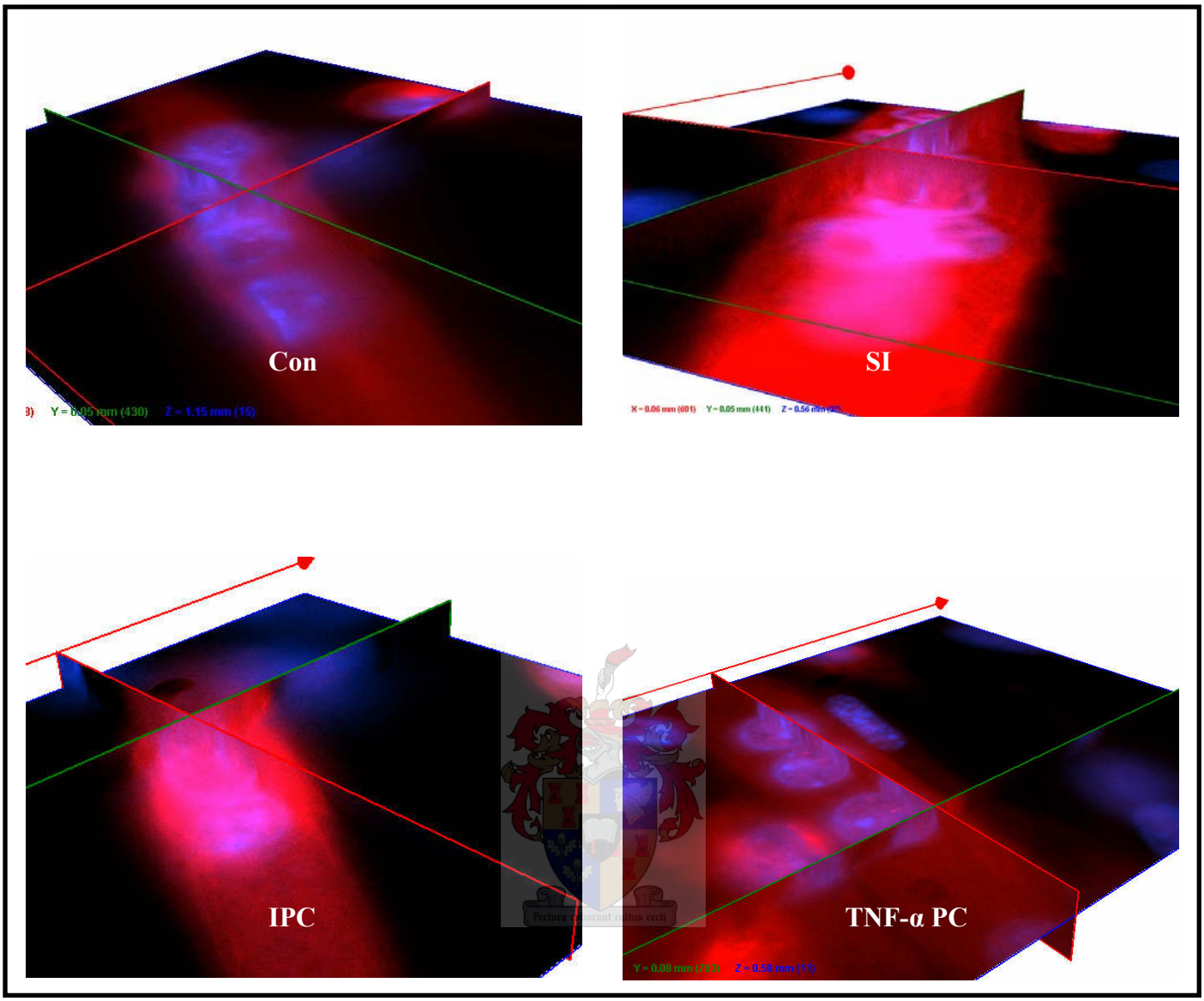


Figure 3.5.2.a. C2C12 myotubes labeled with pcPLA2/TexRed and HOECHST. The figure shows control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC) in slice view with x, y and z spatial dimensions.

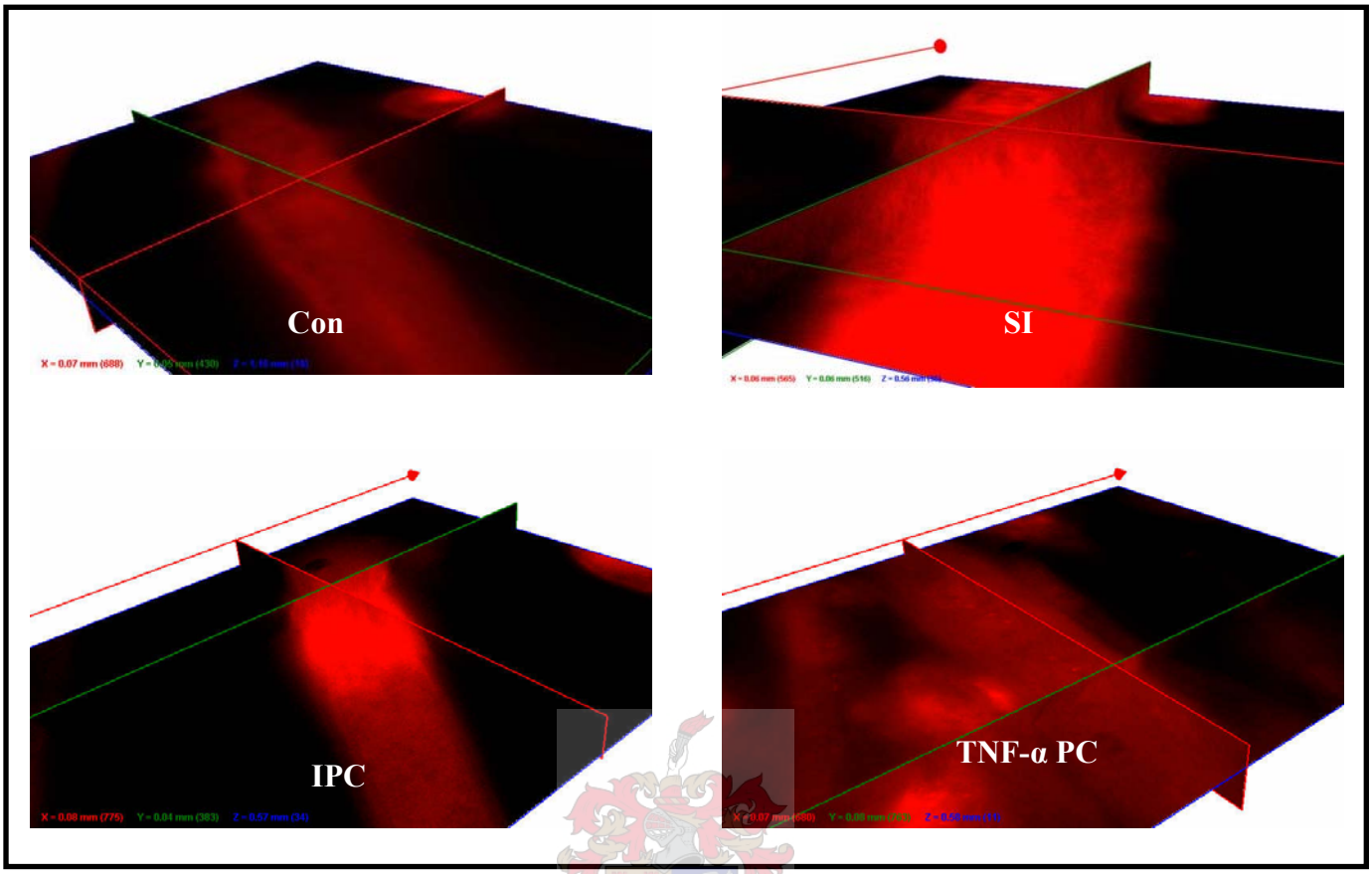


Figure 3.5.2.b. C2C12 myotubes labeled with pcPLA2/TexRed only. The figure shows control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC) in slice view with x, y and z spatial dimensions.

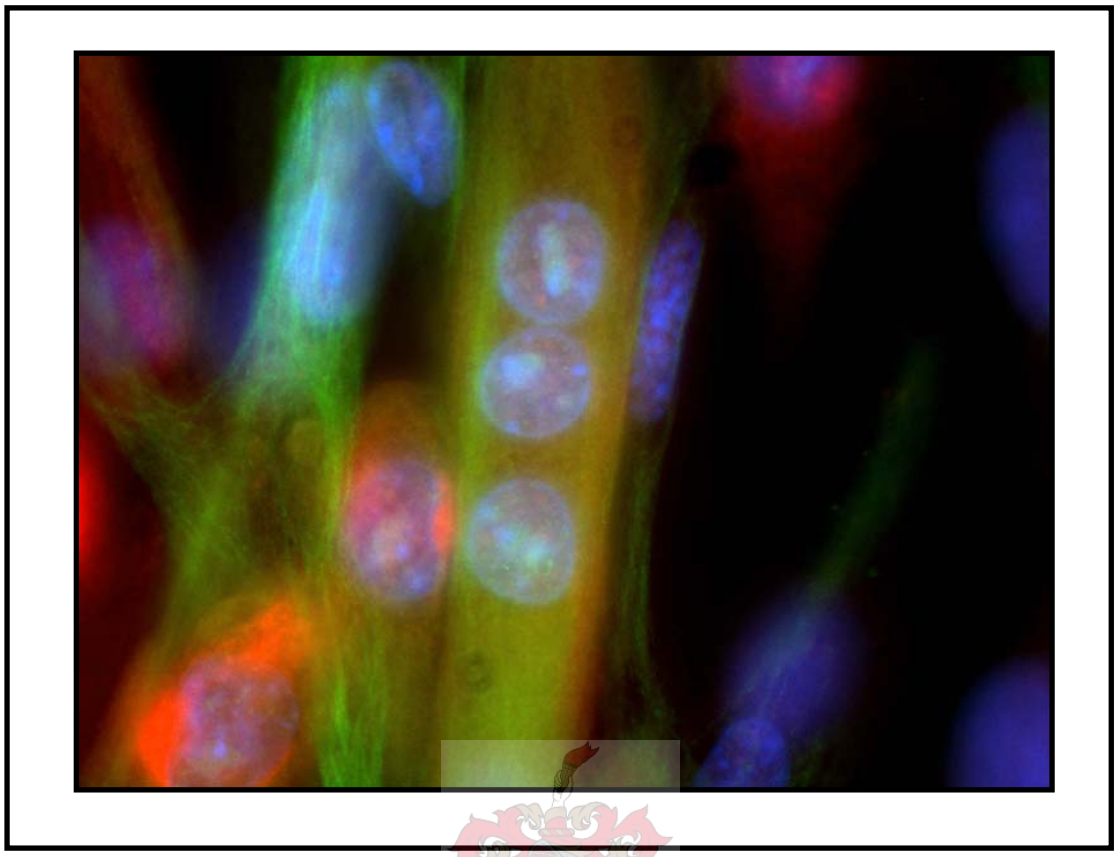


Figure 3.5.3.a. C2C12 myotube labelled for nucleoporin p62, FITC linked. Nucleoporin is displayed in green, indicating the nuclear pores and nuclear network.

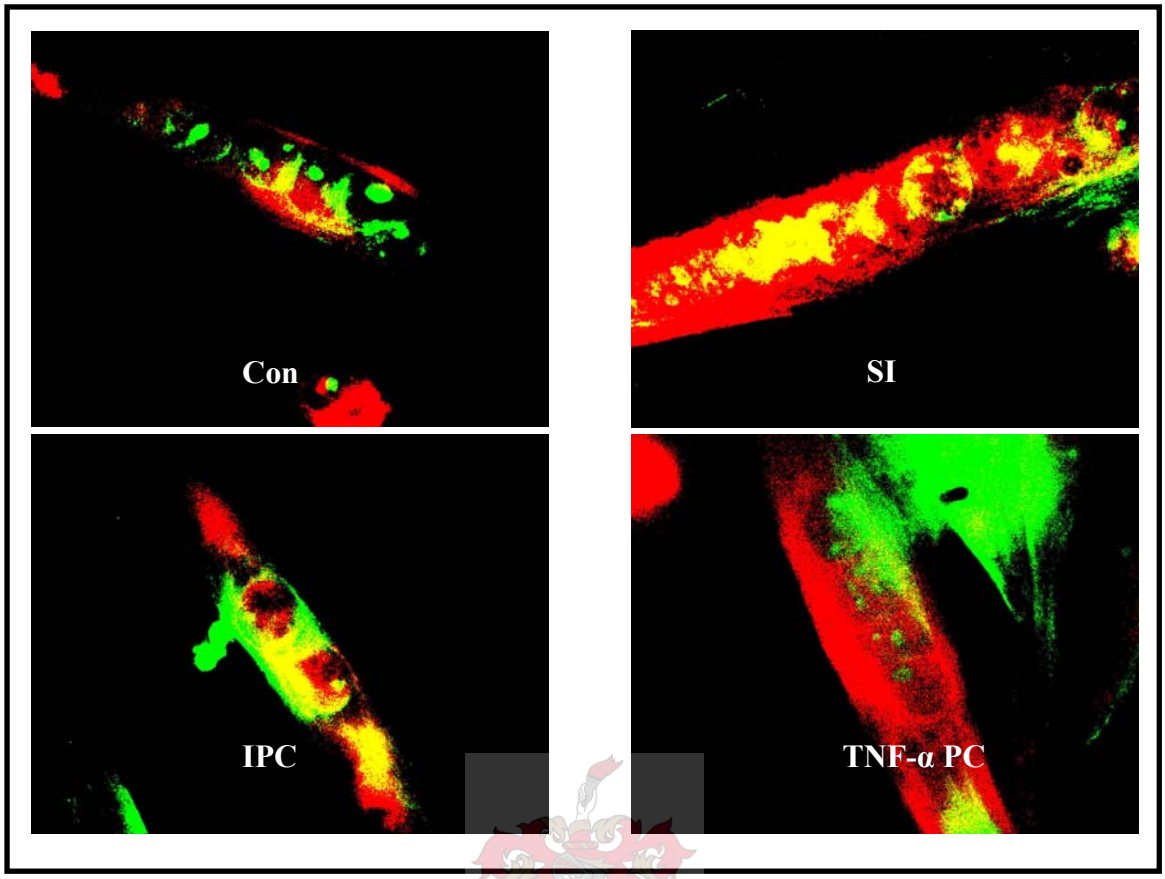


Figure 3.5.3.b. Co-localization of $pcPLA_2$ and nucleoporin p62, based on z-stack imaging at 30 min into the simulated ischaemic event. Displayed are control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC). Co-localization area is displayed in yellow, $pcPLA_2$ in red and nucleoporin p62 in green.

Co-localization area of pcPLA$_2$ and nucleoporin p62

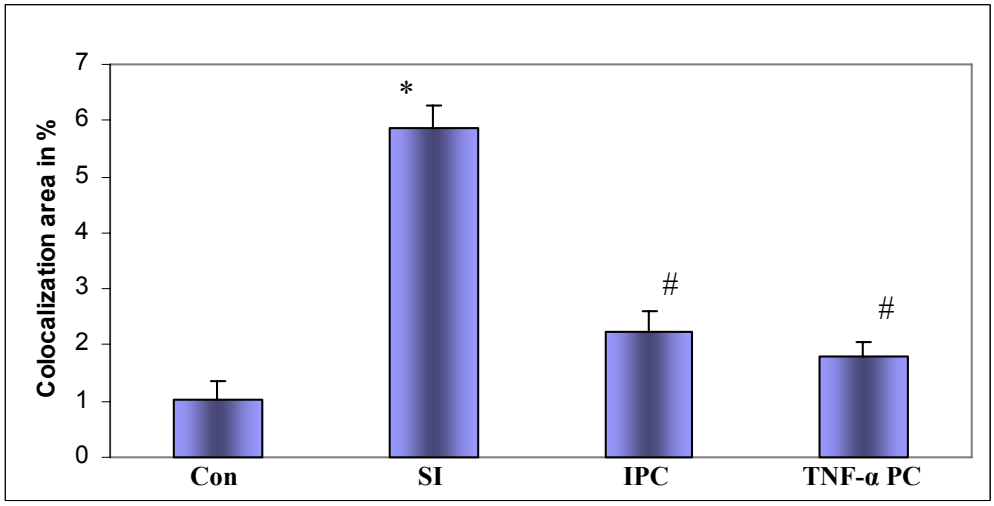


Figure 3.5.3.c. Co-localization area of pcPLA$_2$ and nucleoporin p62, based on z-stack imaging at 30 min into the simulated ischaemic event. Displayed are control (Con), simulated ischaemia (SI), ischaemic preconditioning (IPC) and TNF-α preconditioning (TNF-α PC), * $p<0.05$ vs Con, # $p<0.05$ vs SI, n=3.

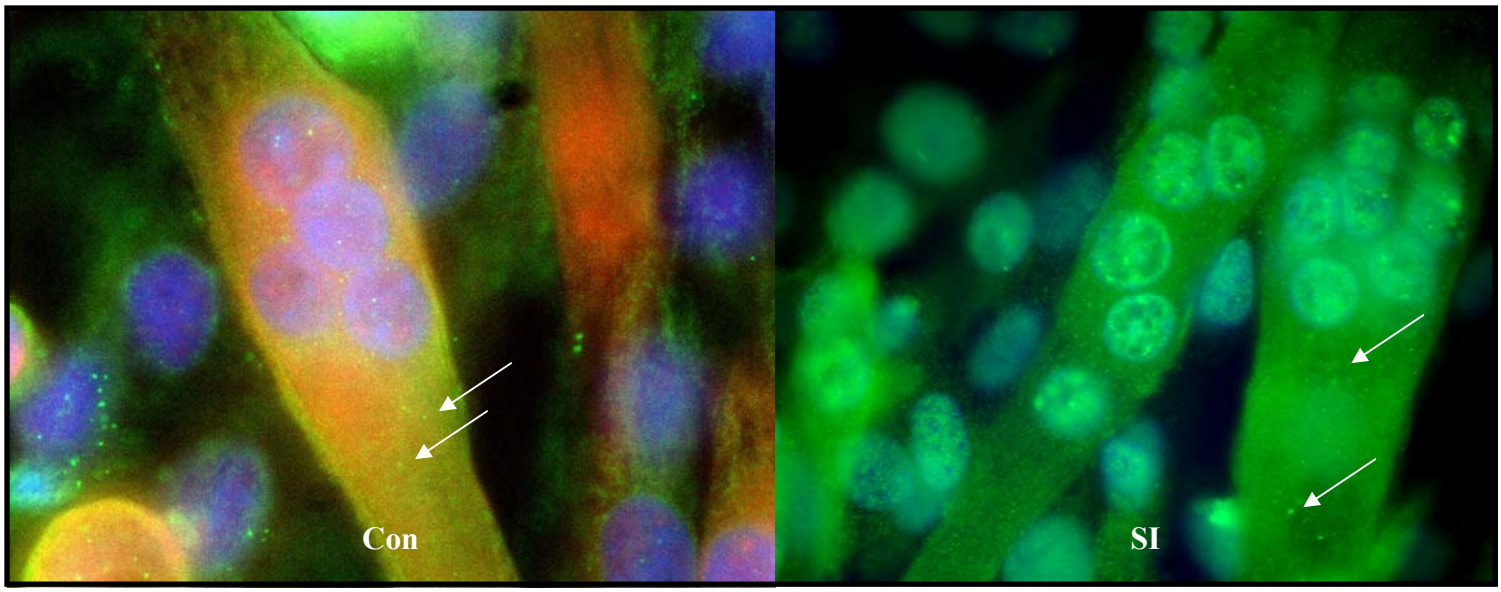
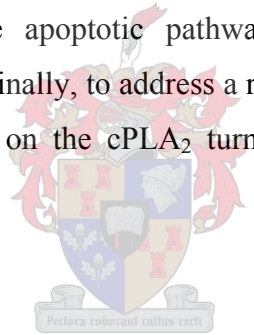


Figure 3.5.4. Labelling control myotubes with the endoplasmic reticulum (ER) marker protein calnexin (green fluorescence signal in left image) results in a clustered or punctuate cytosolic signal pattern. A similar clustered or punctuate pattern can be observed in SI, when staining for pcPLA$_2$ (green fluorescence signal, right image).

Pectora roborant cultus recti

Chapter 4: Discussion

The aim of this study was to examine the involvement of $cPLA_2$ signalling in TNF-α mediated cytoprotection. Therefore, multiple levels of $cPLA_2$ signalling were addressed. Firstly, to assure the cytoprotective effect of the employed low dose TNF-α and to verify the cell line's capacity for ischaemic preconditioning, cell viability assessment was performed. Secondly, as possible contributors to $cPLA_2$ activation, the phosphorylation pattern of p38 MAPK and ERK were investigated at appropriate time points. Thirdly, to dissect out the $cPLA_2$ interplay and dependencies on these MAPKs within the pathway context, the selective $cPLA_2$ inhibitor $AACOCF_3$ was employed and its effect on cell viability was examined. Furthermore, to substantiate $cPLA_2$ activation, we assessed its cellular distribution, translocation and target preference fluorescence microscopically, using co-localization and z-stack techniques. Additionally we examined the apoptotic pathway induction through analysing caspase-3 and PARP cleavage. Finally, to address a recently introduced mechanism of a caspase-3 related modulation on the $cPLA_2$ turnover, we employed the caspase inhibitor, Z-DEVD-FMK.



4.1. Model

In order to investigate the $cPLA_2$ signalling events in the course of ischaemia and TNF-α preconditioning without interference or alterations from other cell types, we made use of a tissue culture model. Our model combines the following properties of ischaemia: Inhibition of glycolysis, which irreversibly prevents the degree of ATP synthesis in the cytosol and reduces the availability of NADH; a significant reduction of the pH of the buffer, inducing a decrease of intracellular pH, similar to that in other models of ischaemia. Additionally, environmental hypoxia of 1% O_2 was used.

The use of differentiated myotubes subjected to simulated ischaemia has some obvious advantages but also some disadvantages compared to *in vivo* ischaemia. The major advantages of this approach are that heterogeneity of ischaemic-induced damage is kept to a minimum, which is of special importance in TNF-α and inflammatory mediator induced signalling, due to induced feed back mechanisms

involving other inflammatory cells. Due to the fact that the C2C12 cell line is an established and recognized model used for cardiac and skeletal muscle research applications, the data can serve a wide purpose. However, our *in vitro* approach functions on cost of physiological parameters which can be maintained and controlled in *in vivo* models. Furthermore, although the C2C12 model is suitable to study molecular mechanisms, the non-contracting nature of the cells makes it less comparable to cardiomyocytes or the isolated perfused heart in terms of ATP demand and work performance. Due to the irreversible character of 2-deoxy-D-glucose, which traps phosphate as 2-deoxy-D-glucose-phosphate, a reperfusion phase would hamper the recovery phase. We therefore decided not to implement a reperfusion phase in this model since it would only be a reoxygenation phase and not a true reperfusion. Furthermore, due to the tendency of nonviable myotubes to detach from the surface, which would be enhanced through any additional change of buffers and washing steps, the harvesting of cells for proteins would be inconsistent and could potentially result in false positive or false negative results. However, since certain pathways and signalling components are only executed at reperfusion, these components could not be taken into consideration for the study. Regarding protein extraction, the fact that non-viable cells possibly leak enzymes due to a loss in membrane integrity, needs to be considered as a disadvantage. The cell lysate sonicated and analysed is therefore a mixture of viable and nonviable cells. Additionally, due to the fact that C2C12 cells have been differentiated from myoblasts into myotubes, there is a degree of a heterogeneous cell population character. The only way of partially compensating for that is highly consistent culture work, using the same techniques and using cells at the same time points and within the frame of a certain passage number only.

4.2. Cell viability

Our data regarding cell viability verify that the methodology employed for the experimental protocol was functional. The ischaemic insult was potent, since 8 hrs simulated ischaemia together with the irreversible glycolysis inhibitor, 2-deoxy-glucose contained in the Esumi buffer, induced an ischaemic event which was sufficient to reduce viability significantly after 8 hrs in the SI group (Figure 3.1.1.). The cytoprotective effect of 0.5 ng/ml TNF-α was verified and reflected in significant

increase in viability compared to SI. Also, the capacity of the cells used in the experiment, to be classically preconditioned, was shown in their increased viability, reaching significance when compared to SI. Although there was no significant difference between the degree of protection in the two preconditioning groups, IPC and TNF-α PC, a tendency towards a greater cytoprotective effect could be observed in TNF-α PC. The selective $cPLA_2$ inhibitor, $AACOCF_3$, also induced significant cytoprotection (Figure 3.1.1.). However, the degree of this cytoprotection was not modulated, when using $AACOCF_3$ in the presence of 0.5 ng/ml TNF-α.

Observations made in several studies are in agreement with these findings, and attribute a cytoprotective effect to $cPLA_2$ inhibition, depending however, on model and cell type employed. In a cerebral ischaemia $cPLA_2^{-/-}$ knock out mice model, a significant reduction in brain infarct volume was shown compared to $cPLA_2^{+/+}$ wild type mice (Sapirstein and Bonventre, 2000). Global ischaemia and the high intracellular levels of free Ca^{2+} resulting from ischaemic membrane depolarisation and opening of Ca^{2+} channels were mentioned as factors to trigger $cPLA_2$ activation (Kramer and Sharp, 1997). Due to alterations in physicochemical properties of the cellular membranes in the ischaemic event, the phospholipid containing membranous structures are made more vulnerable to $cPLA_2$ activity. Kriem *et al.* (2005) demonstrated that in rat cortical neurons, when treated with $cPLA_2$ antisense oligonucleotides and a selective inhibitor of $cPLA_2$ activity, AA release was abolished and cells were protected from apoptosis induced by the amyloid peptide Aβ. Furthermore, the use of inhibitors of ERK and p38, also reduced Aβ induced cell death (Kriem *et al.*, 2005).

In context of the myocardium, although also highly susceptible to an ischaemic insult, we find conflicting results regarding the role of $cPLA_2$. There is evidence that alterations in the $cPLA_2$ activity are able to modulate myocardial AA levels and that the extent of AA accumulation is proportional to the degree of underperfusion or ischaemia (Van der Vusse *et al.*, 1997). Many suggestions have been made to link the $cPLA_2$-induced AA generation with cardiac function and ischaemic tolerance. Peroxidized metabolites of AA could exert inherent toxic effects. It has been shown that the enzymes that metabolize AA produce reactive oxygen species (Burton *et al.*, 1990). This is contextually important since the production and release of reactive

oxygen species produced in ischaemia and reperfusion induce peroxidation of lipid membranes, which in turn increases their susceptibility to the action of $cPLA_2$. This would also imply that the recruitment of the inflammatory pathway is detrimental in this context. Furthermore, AA administration has been shown to affect the amplitude of the Ca^{2+} transient in cardiac myocytes and reduced the contraction rate (Van der Vusse *et al.*, 1997). These AA-linked observations could explain the mechanism of $AACOCF_3$ induced cytoprotection in ischaemia, inducing a shift from the inflammatory pathway. On the other hand, it has been reported that external AA administration to ischaemic neonatal cardiomyocytes provides cytoprotection (Engelbrecht *et al.*, 2005). Gottlieb and co-workers (2002) have shown in the isolated rat heart model, that the calcium independent phospholipase A_2 ($iPLA_2$) is detrimental to the heart and that inhibition of $iPLA_2$ confers cardioprotection. However, employing the $cPLA_2$ inhibitor $AACOCF_3$ in that model, no cardioprotection was observed and it was proposed that $cPLA_2$ is not involved in ischaemia/reperfusion injury. On the other hand, when sepsis is induced and $cPLA_2$ measured in the rat heart, a 60% $cPLA_2$ activity increase was observed during late sepsis, which was related to organ function and mortality (Tong *et al.*, 1998).

These conflicting results could be explained by a differential tissue $cPLA_2$ bioavailability. It has been suggested that the overwhelming majority of measurable phospholipase A_2 activity in the heart is the calcium-independent $iPLA_2$ isoform (McHowat and Creer, 2004). A 10-fold induction of $iPLA_2$ during a brief episode of myocardial ischaemia has been reported (Hazen *et al.*, 1991). In fact, the complexity of the signalling cascade and the mechanism for $cPLA_2$ activation is further complicated by the overlapping expression of multiple PLA_2 enzymes with interdependent actions within the cell. Thus, it has been suggested that $cPLA_2$ might be important in modulating the activity of $iPLA_2$ (McHowat and Creer, 2004). In this context it needs to be mentioned that $AACOCF_3$ is not totally specific to $cPLA_2$, but also potentially inhibits $iPLA_2$. However, it exhibits a 500-fold greater potency against $cPLA_2$ than against $iPLA_2$. The inhibition is likely to be highly complex due to the inter-dependent relationship.

The cytoprotective effects seen with TNF-α PC in this model are in agreement with recent studies. Although there are minor differences in the extent of protection we

observed, compared with data from available literature, this might be attributed to the heterogeneous character of the C2C12 cell line, culture media composition, the percentage of O_2 in the hypoxic chamber and the discrepancies in the time needed to achieve this hypoxic environment.

How can the protective mechanism seen with TNF-α PC be explained? The expression of low concentrations of TNF-α for relatively short periods of time provides the heart with a short term adaptive response to stress. The same is highly likely to be applicable to skeletal muscle tissue. These patterns are mirrored when preconditioning the heart with a low dosage of TNF-α for a short time period. Despite the capacity of TNF-α to function as an ischaemic preconditioning mimetic, a dose dependent effect of TNF-α determined the outcome of tissue tolerance to ischaemia (Lecour *et al.*, 2002). Using recombinant TNF-α as preconditioning stimulus, a dose of 0.5 ng/ml TNF-α was most beneficial in myocardial infarct size reduction following the ischaemic event (Lecour *et al.*, 2002).

The fact that TNF-α contributes to cardiac dysfunction and cardiomyocyte death in I/R injury has led to the investigation of several molecular role players. It has been suggested that H_2O_2-induced p38 MAPK activation may be involved in the TNF-α generation via the activation of NFκB. It was reported that p38 MAPK contributes to TNF-α production during I/R and that inhibition of p38 MAPK attenuates TNF-α production and mitochondrial damage (Kimura *et al.*, 2006). NFκB itself is becoming apparent in the pathophysiology of I/R injury and ischaemic preconditioning, since it can be activated by reactive oxygen species and cytokines. In that way TNF-α can induce NFκB activation, which in turn is able to induce TNF-α gene transcription (Valen *et al.*, 2001). On the other hand it has been shown that in the context of TNF-α mediated cytoprotection, p38-MAPK activation does not contribute to cardioprotection. TNF-α- induced phosphorylation of p38-MAPK was detected only in the non-cardioprotective TNF-α concentrations (Tanno *et al.*, 2003). In the setting of anesthetized rabbits it was shown that antibodies against TNF-α, when given one hour before coronary artery occlusion, were as effective as ischaemic preconditioning in reducing the infarct size. The treatment also abolished the increase in circulating TNF-α levels (Belosjorow *et al.*, 2003).

Our results show no significant differences in viability between IPC and TNF-α PC. The literature suggests that IPC and TNF-α signalling are interlinked via adenosine. It has been shown that adenosine´s anti-inflammatory effects include a decrease in cardiac TNF-α production following I/R injury, as well as the inhibition of neutrophil adhesion to cardiac myocytes (Meldrum, 1998). Ischaemic preconditioning or adenosine stimulation decreased myocardial TNF-α production and improved post-ischaemic functional recovery. These observations suggested a distal effector role for TNF-α production (Meldrum *et al.*, 1998). Cardioprotection by ischaemic preconditioning is associated with reduced TNF-α production during I/R (Belosjorow *et al.*, 1999). When preconditioning is performed with TNF-α, the preconditioning mimetic downregulates its own production. Using TNF-α null (TNF-α $^{-/-}$) mice , it has been shown that cardiac TNF-α production is a necessity for cardioprotection induced by IPC, substantiating a key role of TNF-α in the IPC cascade. Furthermore, administration of a low dose recombinant TNF-α mimicked IPC in wild-type mice, but not in the TNF-α deficient species (Smith *et al.*, 2002). Some of these findings are of course not applicable to the cell culture model used, since here, all systemic effects are excluded. Furthermore, in our viability study one cannot differentiate the contribution of necrosis or apoptosis towards cell death. However, the trypan blue method used in this protocol is based on cellular plasma membrane integrity and function, challenging the exclusion of trypan blue. This mechanism might be more related to necrotic than apoptotic cell death, where the membrane integrity is maintained.

TNF-α-induced cytoprotection from ischaemia has also been shown to occur in other tissues. In neurons it has been shown that TNF-α plays a key role in the ischaemic tolerance induced by ischaemic preconditioning and it was proposed that the sphingolipid, ceramide, is a mediator for this tolerance (Liu *et al.*, 2000). In context of focal cerebral ischaemia, TNF-α was able to induce significant protection against ischaemic brain injury, when used in a low dose (0.5 µg/mouse) as pre-treatment (Nawashiro *et al.*, 1997). Low dose TNF-α pre-treatment prior to hepatic ischaemia has also shown to protect hepatocytes with substantially reduced liver injury (Teoh *et al.*, 2003). The sphingomyelin-ceramide signalling pathway is also activated in response to I/R injury. This is of interest, since sphingomyelinases are activated via the high affinity TNFR1. Resulting ceramide accumulation is implicated in the

production of free radicals, but has been shown to also exhibit a protective role against ischaemic injury (Levade *et al.*, 2001). Others have shown that the infarct limiting effect seen in TNF-α preconditioning was abolished when using a ceramidase inhibitor and that the C2-ceramide itself was able to mimic the preconditioning effect (Lecour *et al.*, 2002).

To substantiate the role of free radicals, it has been shown that when preconditioning with TNF-α, free radical release was substantially increased with the protective low dose, and that the protection was abolished when using a potent antioxidant (Lecour *et al.*, 2005) . It has been suggested that both ischaemic and TNF-α preconditioning are converging at the mitochondria level, the likely source of oxygen radical synthesis (Lecour *et al.*, 2002). Another study showed that TNF-α pre-treatment causes a biphasic increase of ceramide levels and a cellular failure to up-regulate intercellular adhesion molecule 1 (ICAM-1), critical for leukocyte transmigration to the site of inflammation and injury (Ginis *et al.*, 1999). This would suggest a role of inflammation inhibition in the context of TNF-α mediated cytoprotection.

4.3. Apoptosis and $cPLA_2$ phosphorylation

A significant downregulation of $cPLA_2$ in the TNF-α PC group was observed after 8 hrs ischaemia (Figure 3.1.2.). However, a similar degree of $cPLA_2$ phosphorylation was observed in the SI group. Although there is evidence that TNF-α can regulate $cPLA_2$ (Amadou *et al.*, 2002), this does not seem to be the case in our model. This is most probably due to the major variations in the cell types employed in the studies involved. Furthermore, in rat ventricular myocytes a TNF-α-induced stimulation of different PLA_2 isoforms has been demonstrated, and it is known that different tissues show a differential phospholipase profile (Liu and McHowat, 1998). It is interesting that in our study, IPC completely reduces $cPLA_2$ phosphorylation, reaching statistical significance compared to the SI group (Figure 3.1.2.). Similar results are seen when employing the caspase inhibitor Z-DEVD-FMK. Z-DEVD-FMK is an irreversible cell-permeable inhibitor of caspase-3, -6, -7, and -8. However, when using the inhibitor in the presence of 0.5 ng/ml TNF-α, the observed inhibition was overridden, resulting in a significantly higher degree of $cPLA_2$ phosphorylation. According to the protein band pattern, we do not find any indication for $cPLA_2$ cleavage. Total $cPLA_2$

levels were constant and no additional bands of lower molecular weight, which indicate cleaved protein fragments, were observed. Thus, our results do not conform with the proposed mechanism of caspase induced $cPLA_2$ cleavage (Kronke and Adam-Klages, 2002). The multiple cleavage sites on $cPLA_2$ for various caspases would ensure its cleavage, avoiding the induction of the inflammatory pathway and assuring apoptosis. Indeed, it has been shown that cleavage of $cPLA_2$ results in the inactivation of its enzymatic activity (Kronke and Adam-Klages, 2002). In TNF-α stimulated HEK cells an increase in $cPLA_2$ activity was seen after caspase-3 inhibition, suggesting a role for caspases to downregulate or terminate $cPLA_2$ activation. This could explain the observed increased viability seen when employing the $AACOCF_3$ inhibitor. However, as discussed at a later stage, our images suggest that a differential translocation pattern is associated with the TNF-mediated cytoprotection, mediating a functional $cPLA_2$ inhibition, and thus inhibiting the inflammatory pathway.

It can be seen in the caspase-3 cleavage pattern, that the apoptotic pathway in our model, following 8 hrs ischaemia, is activated (Figure 3.1.3.). As anticipated, the caspase inhibitor significantly decreased caspase-3 cleavage, but it was overidden when co-incubating with TNF-α. However, TNF-α PC shows a significantly higher degree of caspase-3 cleavage than IPC. Viability investigations will help to interpretate these findings in context. This is of importance, since the apoptotic pathway, though activated, is not necessarily executed. Thus, the induction of apoptosis could increase the chance of cell survival, as long as the pathway is still reversible. This phenomenon could function similarly to IPC, namely *buying out lethal time*. The only challenge remains, is to dissect out the time frame when ischaemic injury is still reversible.

Our western blot results show, that caspase-3 cleavage is induced during SI (Figure 3.1.3.). It has been proposed that apoptosis may be the prominent form of ischaemia-related cell death in the heart. This, however, should only make sense at the onset of ischaemia, when one considers the extremely high ATP demand in the myocardium, which would lead to rapid ATP depletion and would cause a shifts towards necrosis induction (Clerk *et al.*, 2003). It would be highly feasible for the ischaemically challenged cell to extend and maintain the apoptotic pathway and thereby increasing

the chance of apoptosis reversibility upon reperfusion. It was verified in a TNF-α resistant L929 cell line, deficient in $cPLA_2$, that $cPLA_2$ plays a key role in TNF- α-induced apoptosis. When $cPLA_2$ expression was induced, the TNF- α response could be restored (Taketo and Sonoshita, 2002). It was also recently shown by Fu and co-workers (2004) that TNF-α reduction in the cardiomyocyte could have an impact on the caspase-3 pathway, and when employing neutralizing anti-TNF-α antibodies, norepinephrine induced apoptosis was attenuated (Fu *et al.*, 2004).

As discussed in chapter one, cleaved caspase-3 is able to cleave the enzyme PARP. The signal for the intact 116 kDa PARP molecule was detectable in untreated normoxic control cells where the 116 kDa band was strong (Figure 3.1.4.). In treated cells, the weak 116 kDa signal indicated PARP cleavage. We observed that the extent of caspase-3 cleavage was mirrored by the extent of PARP cleavage, seen in the reduction of the total PARP 116 kDa molecule, after 8 hrs ischaemia. This might be indicative of the progression of apoptosis induction within the pathway.

Unfortunately the caspase-3 silencing and transfection was not successful, reflected in a caspase-3 band similar to that of the control cells (for purposes of documentation, Figure 3.1.5. is included). This is most probably related to the known difficulty in transfecting muscle cells, since a transfection reagent applicable for a broad range of cells was used. Nevertheless, the western blot results found at the 8 hrs time point and the viability data made us decide to investigate the signalling mechanism of $cPLA_2$ early in the simulated ischaemic event.

4.4. Cellular signalling

It was decided that 5 min and 30 min into the ischaemic event would be the most appropriate time points to elucidate signalling events in our cell model, due to the likelihood of $cPLA_2$- and MAPK phosphorylation (van Rossum *et al.*, 2004).

To our surprise, we did not find any significant differences regarding $cPLA_2$, p38- and ERK phosphorylation at 5 min into ischaemia (Figure 3.2.1., Figure 3.2.2.a./b.). A faint banding pattern implies a very small degree of $cPLA_2$ phosphorylation under control conditions. We also observed a faint and non-significant pERK and pp38

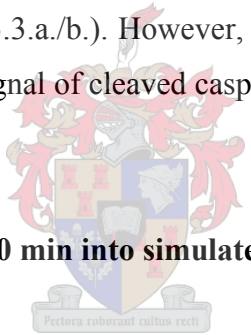
signal in the control cells, which could be attributed to the serum-containing culture medium (van Rossum *et al.*, 2001). It has been shown that the mechanisms involved in the regulation of $cPLA_2$ such as the phosphorylation, calcium concentration and site of subcellular location appear to be cell-type and agonist-dependent (Gijon *et al.*, 1999). The differences in potency and effect of the simulated ischaemic event might account for these findings. We also could not describe an effect of the $cPLA_2$ inhibitor on the phosphorylation state of these kinases. Furthermore, we did not observe significant changes in the caspase-3- or PARP cleavage pattern (Figure 3.2.3.a./b.).

We still did not see any changes in the $cPLA_2$ phosphorylation pattern at 30 min into the ischaemic event (Figure 3.3.1.). This is surprising since other data suggest phospholipase activation early in ischaemia. It has been shown that ischaemia affects phospholipid metabolism in a profound way, with increasing susceptibility of phosphatidylcholine synthesis, in the isolated rat heart model (Lochner and de Villiers, 1989). Here it was demonstrated, that already within 10 min, the incorporation rate of (methyl-3H) choline into tissue phospholipids was significantly inhibited, whereas tissue choline levels remained the same (Lochner and de Villiers, 1989).

Although there was an increase in ERK phosphorylation in the IPC and TNF-α PC groups compared with Con and SI, it was not significant. Also, the $cPLA_2$ inhibitor, $AACOCF_3$, caused a significant increase in ERK phosphorylation, compared to control values (Figure 3.3.2.a.). This is in agreement with our viability results, since ERK is known to mediate survival signals in the cell (Burgering and Bos, 1995). Several studies indicated a role for ERK in IPC (Omura *et al.*, 1999; Liu *et al.*, 2003). In response to a preconditioning stimulus, ERK has been shown to translocate to the nucleus, where it has been demonstrated to activate NFkB and AP-1 (Hobbie *et al.*, 1997; Hausenloy and Yellon, 2006). Activation of ERK-1/2 at reperfusion, following a prolonged ischaemic event, protects the heart against ischaemia/reperfusion injury (Hausenloy *et al.*, 2004). However, in the context of TNF-α PC, it has been shown that ERK activation in the early reperfusion phase is not essential for TNF-α induced preconditioning (Lecour *et al.*, 2005).

Unexpectedly, our results indicated a significant increase in p38 phosphorylation only in the IPC group, with a trend towards increased p38 phosphorylation in the TNF-α PC group (Figure 3.3.2.b.). This is in agreement with results from Weinbrenner *et al.* (1997), who showed that p38 MAPK activation correlates with the protection induced by preconditioning in rabbit hearts (Weinbrenner *et al.*, 1997)**.** Furthermore, attenuation of p38 MAPK activation during ischaemia and during reperfusion was associated with improved functional recovery during reperfusion (Marais *et al.*, 2001). p38 MAPK activation has been shown to be induced by a multi-episode preconditioning protocol which was associated with a significant reduction in p38 MAPK activation at global ischaemia and at reperfusion, compared with the marked activation observed in untreated non-preconditioned hearts. In many cell types p38 also appears to enhance apoptosis (Park *et al.*, 2000).

At 30 min into ischaemia we do not observe significant changes in the caspase-3 or PARP cleavage pattern (Figure 3.3.a./b.). However, the trend already observed at the 5 min time point, with a lower signal of cleaved caspase-3 in the control and SI group, is mirrored also at 30 min.



4.5. Immunocytochemistry at 30 min into simulated ischaemia

4.5.1. $cPLA_2$ translocation

A functional phospholipid bilayer system such as the sarcolemma is crucial to maintain cellular function. Not only is the intact cell membrane needed to control ion homeostasis, but membrane integrity is also necessary to provide an optimal receptor platform, with a defined membrane fluidity to allow optimal receptor mediated pathway interactions. Another important role of phospholipid bilayers is their contribution to subcellular organelle membranes, such as mitochondria, the endoplasmic reticulum (ER), the Golgi apparatus or the nuclear network, contributing to a functional compartmentalization within the cell. The subcellular membranes provide a microenvironment needed for each organelle to fulfil its specific function. Via hydrolization of fatty acyl moieties of membrane phospholipids through extensive actions of $cPLA_2$, this environment would be disturbed, and its optimal functional capacity reduced.

However, an ischaemically challenged cell undergoing necrosis loses its membrane integrity, with detrimental consequences for the cellular environment. One important role player in this context is the activation of phospholipases and specifically $cPLA_2$, as it has been suggested as a likely candidate in the pathogenesis of ischaemia and reperfusion injury (Van der Vusse *et al.*, 1997). Membrane-associated enzyme systems such as the ATP-dependent Ca^{2+} transporter can be impaired due to the activation of $cPLA_2$ (Tong *et al.*, 1998).

We, therefore, investigated the translocation pattern of $cPLA_2$ at 30 min into the ischaemic event, a relevant time point as it allows time to provide stimulatory signals for $cPLA_2$ activation (van Rossum *et al.*, 2004). Our results show that in untreated normoxic control cells, staining for $pcPLA_2$ resulted in a fine punctuate labelling pattern randomly distributed throughout the cytoplasm of the myotube, with a fine pattern in the nucleus. The random nature of the distribution was supported by the fact that optical z-sections revealed the pattern in all depths in the cell. However, in SI a significant increase in mean nuclear intensity is observed, indicating $cPLA_2$ translocation to the nucleus and peri-nuclear region. Both the IPC and TNF-α PC group showed a significant increase in nuclear intensity compared to control levels; however these were significantly less than in the SI group. These findings were verified using two different analysis techniques and different labelling techniques. Our z-stack-based analyses showed that intranuclear speckles are present, with the highest fluorescence intensity seen in SI, suggesting an association of $cPLA_2$ with invaginations of the nuclear membrane (Grewal *et al.*, 2005). In context of the inflammatory pathway, enzymes such as COX, which are situated downstream of $cPLA_2$, and which are involved in eicosanoid synthesis, are localized in the nuclear membrane region (Murakami *et al.*, 2003). An emerging body of evidence suggests that the spatio-temporal co-localization of eicosanoid-biosynthetic enzymes in the perinuclear region is critical for their effective functional coupling (Pardue *et al.*, 2003). COX-1 is generally considered as a house keeping enzyme that is constitutively expressed and involved in homeostasis. The inducible COX-2, on the other hand, is expressed primarily in disease states and in response to inflammation. Both isoforms have been shown to localize at the nuclear envelope and endoplasmic reticulum (Grewal *et al.*, 2005). It is thought that the liberation of free AA is rate

limiting for the synthesis of these mediators. Furthermore, AA release has been found to be proportional to the extent of $cPLA_2$ translocation and to the Ca^{2+} concentration in the cell (Szabo and Dawson, 1998).

It can therefore be hypothesized that the observed translocation of $cPLA_2$ to the nuclear region leads to AA generation and functional enzyme coupling, leading to the generation of inflammatory mediators. Furthermore, due to the differential translocation response seen in our results one can expect a differential enzyme induction and metabolite accumulation. This in turn is critical in subcellular loci such as the perinuclear region and could lead to alterations in subcellular membrane molecular dynamics, thus precipitating membrane dysfunction (Hazen *et al.*, 1991). Our results imply that IPC and TNF-α PC inhibit $cPLA_2$ relocation, keeping it to a large extent in the cytoplasmic compartment and therefore possibly inhibiting functional $cPLA_2$ activity (Figure 3.5.1.c.). These findings imply that both TNF-α PC and IPC lead to a decrease in inflammatory mediator production, and hence a shift away from the inflammatory pathway. This might contribute to the cytoprotective effect observed in these groups.

The results clearly indicate that the sarcolemmal membrane *per se* is not involved as target phospholipid bilayer for $cPLA_2$ under ischaemic conditions, but rather that phospholipid components in the nuclear envelope and network region are favoured. Interestingly, the intermediate filament vimentin has been suggested to serve as functional adapter for $cPLA_2$, acting as a scaffold protein and assisting in appropriate interactions between $cPLA_2$ and perinuclear phospholipids membranes to stabilize the binding of $cPLA_2$ to the membrane (Murakami *et al.*, 2000). That the target organelle is crucial in terms of cellular outcome has been shown in several studies (Evans *et al.*, 2001). Bunt and co-workers (1997) showed a similar punctuate labelling pattern of $cPLA_2$ in fibroblasts under normoxic conditions (Bunt *et al.*, 1997). Others (Herbert *et al.*, 2005) have shown that $cPLA_2$ activity and function in controlling endothelial cell proliferation is regulated by reversible association with the Golgi apparatus.

Differential Ca^{2+} dependent membrane targeting of $cPLA_2$ has been described with various intracellular organelles, including the ER, the perinuclear region and the Golgi apparatus (Evans *et al.*, 2001). It is of interest to try to explain this differential

$cPLA_2$ translocation pattern in our cell model. It is known that under ischaemic conditions the Ca^{2+} handling capacity of the cell is impaired (Hearse, 1994). Ca^{2+} is the crucial activation stimulus for $cPLA_2$ (Clark *et al.*, 1995). The observed translocation pattern could therefore be an indicator for the cell's Ca^{2+} handling capacity. Preconditioning has been described as a mechanism to lessen calcium-mediated damage (Opie, 1997). There are also data available which show that TNF-α can modulate the sarcoplasmic reticulum Ca^{2+} release and Ca^{2+} transients in the ischaemic myocardium (Meldrum *et al.*, 1998). $cPLA_2$ modulates ER calcium handling and might contribute to ineffective calcium sequestration by the sarcoplasmic reticulum, rather than an ineffective sarcolemmal calcium extrusion. Since the same reduction in $cPLA_2$ translocation is seen in IPC, better calcium handling might functionally inhibit $cPLA_2$ and thus contribute to the increased viability seen in IPC, TNF-α PC as well as after treatment with the $cPLA_2$ inhibitor, $AACOCF_3$. In the sarcoplasmic reticulum and other subcellular regions, energy dependent mechanisms take place, such as the activity of ATP dependent Ca^{2+}-ATPase. These mechanisms would be impaired in ischaemia due to the decrease in ATP availability, contributing to impaired Ca^{2+} homeostasis. On the other hand, in this model no contraction takes place as in the case of cardiomyocytes, so the Ca^{2+} dependency and ATP demand differ. However, it can only be speculated whether these mechanisms play a role in our cell model.

4.5.2. $cPLA_2$ co-localization

z-stack experiments are a necessity for co-localization studies, since unlike in a conventional two-dimensional image overlay, all three spatial dimensions are considered and this enables a true calculation for co-localization. For verification of $cPLA_2$ nuclear translocation, the nuclear marker nucleoporin p62 was used and co-labelled with $pcPLA_2$. The results show differential co-localization with the significantly largest co-localization area in the SI group compared to the control cells. Co-localization area in IPC and TNF-α PC was significantly smaller compared to the SI group. These results indirectly mirror the $cPLA_2$ translocation results described above, and substantiate the observed differential nuclear preference.

Interestingly, after careful image analysis we observed, only in the SI group, a different and distinct strong clustered $cPLA_2$ pattern in the cytoplasm. We, therefore, labelled the myotubes for the endoplasmic reticulum (ER) marker calnexin. The images resulted in a similar punctuate cluster pattern, providing preliminary and novel evidence for a distinct and differential target organelle for $cPLA_2$ in SI, namely the ER in addition to the nuclear region.

4.6. Conclusion and future directions

The results provide evidence that there is a role for $cPLA_2$ signalling in TNF-α mediated cytoprotection. The aim of the study was to investigate the kind of $cPLA_2$ involvement, its cellular activation, translocation and degradation, and thus turnover in TNF-α PC.

Although we did not observe a differential activation pattern in TNF-α PC in terms of $cPLA_2$ phosphorylation and no differential inactivation of $cPLA_2$ via cleavage, we describe a differential $cPLA_2$ translocation pattern, similar to that in IPC. We hypothesize that through inhibition of $cPLA_2$ translocation a potential functional $cPLA_2$ inhibition is achieved. This would imply inhibition of the inflammatory pathway, subsequently less generation of inflammatory mediators and a shift away from the inflammatory pathway (Figure 4.6.). Such a mechanism would allow more time for cellular decision making, which otherwise would have been spent committing to irreversible necrotic cell death.

In conclusion, we provide evidence to indicate that TNF-α preconditioning is associated with distinct $cPLA_2$ signalling which may be an important component in the cascade of events in channelling the production of injurious mediators in inflammation. These findings can provide new ideas in the context of inflammation treatment through agents which control differential $cPLA_2$ trafficking within the cell. Understanding the mechanisms underlying these processes has the potential to extend current strategies and to identify new ones to treat cardiac disease. Ischaemic preconditioning has not materialized in the clinic because of the prerequisite for it to be applied prior to the onset of index ischaemia or myocardial infarction, which is

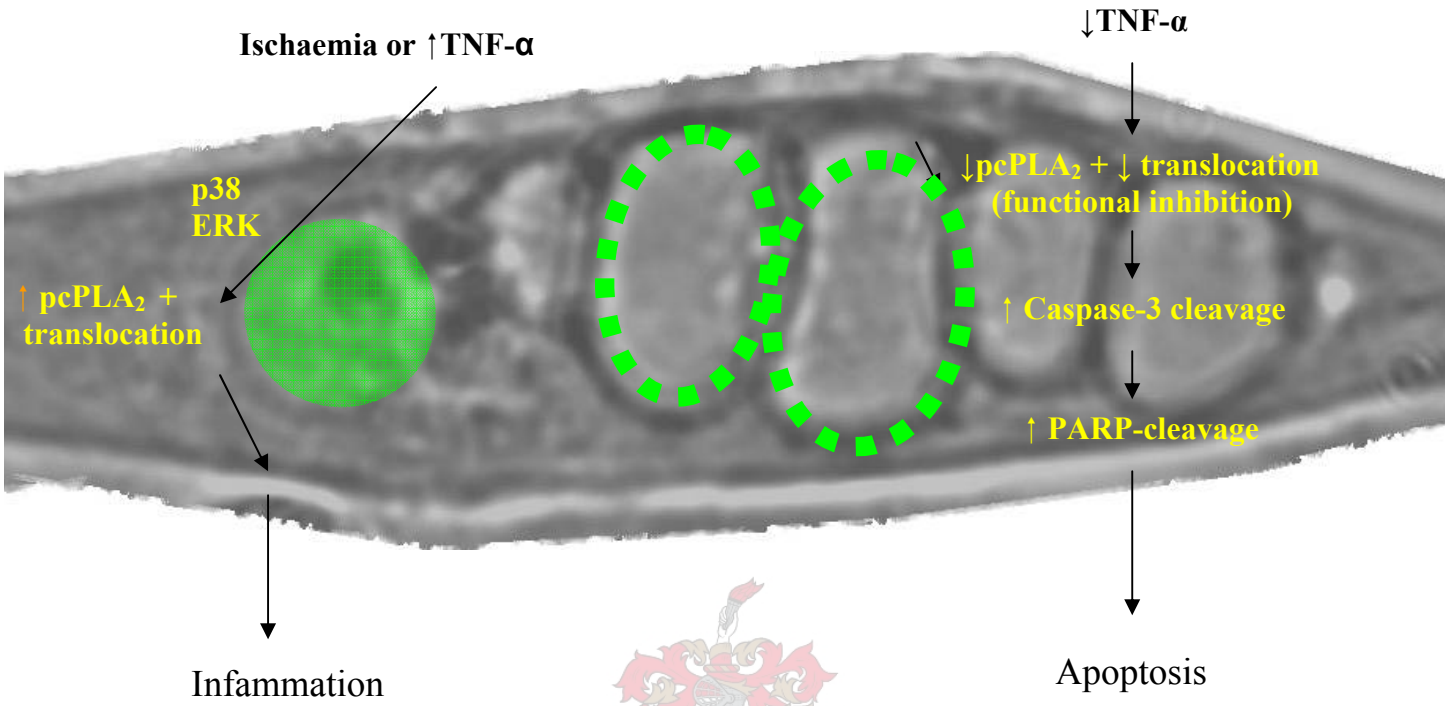


Figure 4.6. Proposed mechanism of $cPLA_2$ associated pathway induction between inflammatory and apoptotic pathway, associated with the extent of cytoprotection.

unlikely to be predictable. However, new strategies employing the growing body of knowledge generated in the field of IPC have recently emerged, which led to the introduction of the post-conditioning concept (Tsang *et al.*, 2004; Yellon and Hausenloy, 2005; Yellon and Opie, 2006). This could also be beneficial in the context of TNF-α preconditioning, providing room for translation of current knowledge into a clinically more relevant context. For example, a longer-termed TNF-α treatment in addition to standard preventative therapy could be used in conditions of ischaemic heart disease and heart failure, to prevent the development of a beneficial environment for inflammation. At the same time this could provide a protective window for a possible future ischaemic event, a fact which is even more desirable, since a weakened and infarcted myocardium is already exposed to a higher level of stress and is shifted out of its homeostatic balance.

For future experiments it would be of great benefit to employ novel techniques to be able to generate data which accurately reflect the real scenario in the ischaemically challenged cell or tissue. It is also necessary to identify the downstream events following $cPLA_2$ activation, like target proteins or metabolic pathways for the liberated AA or other fatty acids. Furthermore, the recruitment of human cells or tissues is suggested, to exclude species dependent discrepancies. For example, the fact that there are double the number of phosphorylation sites in human $cPLA_2$ compared with murine $cPLA_2$, might contribute to a much greater degree of control in the mechanism of $cPLA_2$ activity, possibly with a steeper gradient of recruitable activity (Kramer and Sharp, 1997). Using live cell dyes for relevant cellular organelles such as the ER-tracker or mito-tracker, questions about AA induced ROS production can be addressed under real time conditions. In this context, ion fluorophores such as Fura could be employed to investigate the previously discussed cellular Ca^{2+} handling capacity under real time ischaemic conditions. This will shed more light on the survival mechanisms employed by the ischaemically challenged cell in a setting of TNF-α mediated cytoprotection.

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